Direct Response of the *Escherichia coli* DnaK/DnaJ/GrpE Chaperone System to Heat Shock

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Summary

Molecular chaperones are abundant proteins found in all living organisms. They assist the folding and refolding of nascent and denatured proteins, respectively, both under normal growth and heat shock conditions. DnaK, a member of the 70-kDa heat shock protein (Hsp70) family in *Escherichia coli*, processes its substrates in an ATP-driven cycle, which is controlled by the two co-chaperones DnaJ and GrpE. DnaJ, an Hsp40 homolog, stimulates the hydrolysis of DnaK-bound ATP and converts DnaK from its ATP-liganded T state with low affinity for substrate into the ADP-liganded high-affinity R state, while GrpE facilitates ADP/ATP exchange and reconverts high-affinity R-state DnaK into its low-affinity T state. The fraction of high-affinity R-state DnaK, and thus of sequestered substrate, is controlled by the conjoint action of the two co-chaperones.

The well-known heat shock response in *E. coli* is mediated by the transcription factor σ^{32} and results in enhanced expression of heat shock proteins. Recently, GrpE has been found to undergo a reversible conformational transition in the physiologically relevant temperature range, which decreases its nucleotide exchange activity at higher temperatures (Grimshaw et al., 2001; Groemping and Reinstein, 2001; Gelinas et al., 2002; Moro and Muga, 2006). Apparently, GrpE acts as a thermosensor of the DnaK/DnaJ/GrpE chaperone system. The primary thermosensitive structural element in the GrpE dimer is a pair of long α-helices (Grimshaw et al., 2003; Gelinas et al., 2003).

In the first part of the dissertation, we demonstrate that the thermal transition in GrpE is of functional significance in the complete DnaK/DnaJ/GrpE chaperone system. A mere increase in temperature from 25°C to 45°C resulted in a higher fraction of fluorescence-labeled peptide substrate being sequestered by DnaK. The dissociation equilibrium constant of the DnaK·peptide complex did not significantly change within this temperature range; the observed increase in the fraction of DnaK-bound peptide can thus not be explained by a

temperature-dependent change in the substrate-binding properties of DnaK itself. The temperature-induced shift of DnaK towards its high-affinity R state proved to be fully reversible and thus to be controlled by the thermosensor function of GrpE. When GrpE was omitted in the experiments, no shift of DnaK was observed.

In the second part of the thesis, we show that the thermosensor control of GrpE is essential for optimum chaperone action of the DnaK/DnaJ/GrpE system during heat shock. We compared the performance of wild-type GrpE as component of the chaperone system with that of GrpE(R40C). In this mutant, the thermosensing helices are stabilized with an intersubunit disulfide bond; consequently, its nucleotide exchange activity increases continuously with increasing temperature (Grimshaw et al., 2003). Wild-type GrpE with intact thermosensor proved clearly superior to GrpE(R40C) with desensitized thermosensor. The chaperone system with wild-type GrpE protein protected protein substrate more efficiently against both denaturation and aggregation during heat shock.

In the last part, we show that DnaJ as non-native-protein sensor and GrpE as thermosensor directly adapt the operational mode of the DnaK system to heat shock conditions and promote dynamic sequestering of aggregation-prone proteins. The *cis*-effect of DnaJ on DnaK in ternary (ATP·DnaK)·protein·DnaJ complexes (Han and Christen, 2003, 2004), which accelerates ATP hydrolysis, provides an efficient triage mechanism, selecting from the (ATP·DnaK)·substrate complexes those to be processed in the chaperone cycle; at 45°C, the fraction of protein complexes fed into the cycle is 20 times higher than that of peptide complexes. The thermosensor effect of the ADP/ATP exchange factor GrpE retards the release of substrate from the cycle at higher temperatures; the fraction of total DnaK in stable (ADP·DnaK)·substrate complexes is 2 times higher at 45°C than at 25°C. Moreover, we introduce fluorescence-labeled DnaK H541C as a suitable means for following the binding and release of protein substrates to and from DnaK in real time.

Zusammenfassung

Molekulare Chaperone sind in den Zellen aller Organismen in reichlichen Mengen vorhanden. Sie unterstützen sowohl die Faltung von Polypeptidketten während deren Synthese als auch die Rückfaltung denaturierter Proteine, die besonders bei zellulärem Stress wie einem Hitzeschock auftreten. DnaK ist ein Chaperon in *Escherichia coli* und gehört zur Familie der 70-kDa Hitzeschockproteine (Hsp70). Um als Chaperon zu wirken, interagiert DnaK mit seinen Proteinsubstraten in einem ATP-getriebenen Zyklus, der durch zwei Co-Chaperone, DnaJ und GrpE, gesteuert wird. DnaJ beschleunigt die Hydrolyse von DnaK-gebundenem ATP und versetzt DnaK in seinen ADP-ligandierten R-Zustand, der durch eine hohe Substrataffinität gekennzeichnet ist, während GrpE den Austausch von ADP durch ATP erleichtert und DnaK in seinen ATP-ligandierten, niederaffinen T-Zustand zurückbringt. Der Anteil an hochaffinem DnaK im R-Zustand und damit auch an DnaK-gebundenem Substrat, wird durch DnaJ und GrpE gemeinsam reguliert.

Die allgemein bekannte Hitzeschockantwort in *E. coli* besteht in einer erhöhten Expression von Hitzeschockproteinen und wird durch den Transkriptionsfaktor σ^{32} vermittelt. Kürzlich wurde gezeigt, dass GrpE eine reversible temperaturabhängige Konformationsänderung durchläuft, welche im physiologisch relevanten Temperaturbereich liegt (Grimshaw et al., 2001; Groemping and Reinstein, 2001; Gelinas et al., 2002; Moro and Muga, 2006). Die Konformationsänderung in GrpE erniedrigt bei erhöhten Temperaturen dessen Aktivität als ADP/ATP-Austauschfaktor. GrpE wirkt dadurch als Thermosensor im DnaK/DnaJ/GrpE-System. Das primäre temperaturempfindliche Strukturelement im GrpE-Dimer ist ein Paar von zwei langen, parallel nebeneinander liegenden α-Helices (Grimshaw et al., 2003; Gelinas et al., 2003).

Der erste Teil der Dissertation zeigt, dass die temperaturabhängige Konformationsänderung in GrpE für das DnaK/DnaJ/GrpE-System von funktioneller

Bedeutung ist. Ein blosser Temperaturanstieg von 25°C nach 45°C erhöhte den Anteil an fluoreszenzmarkiertem Peptid, der an DnaK gebunden war. Die Gleichgewichtsdissoziationskonstante des DnaK·Peptid-Komplexes änderte sich in diesem Temperaturbereich nur unwesentlich; der beobachtete Anstieg von DnaK-gebundenem Peptid kann daher nicht durch temperaturabhängige Änderungen der Substratbindungseigenschaften von DnaK selbst erklärt werden. Die temperaturinduzierte Verschiebung von DnaK zum hochaffinen R-Zustand erwies sich als vollständig reversibel und damit als Folge der Thermosensorfunktion von GrpE. Wenn GrpE in der Reaktionslösung weggelassen wurde, konnte keine Verschiebung von DnaK beobachtet werden.

Der zweite Teil dieser Arbeit zeigt, dass die Thermosensorfunktion von GrpE unentbehrlich für die optimale Wirkung des DnaK/DnaJ/GrpE-Systems während eines Hitzeschocks ist. Wir verglichen die Leistung von Wildtyp-GrpE als Bestandteil des Chaperonsystems mit derjenigen von GrpE(R40C). In diesem mutierten Protein waren die temperaturfühlenden α-Helices mit einer Disulfidbrücke stabilisiert worden; als Folge ergab sich mit zunehmender Temperatur ein kontinuierlicher Anstieg der ADP/ATP-Austausch-Aktivität (Grimshaw et al., 2003). Wildtyp-GrpE mit intaktem Themosensor erwies sich der GrpE(R40C) Mutante als klar überlegen. Das Chaperonsystem mit Wildtyp-GrpE schützte die Proteinsubstrate sowohl effizienter gegen Denaturierung als auch Aggregation während des Hitzeschocks.

Der letzte Teil zeigt, dass DnaJ als Sensor für ungefaltete Proteinsubstrate und GrpE als Thermosensor den DnaK-Zyklus direkt an Hitzeschockbedingungen adaptieren und ein dynamisches Sequestrieren von Proteinsubstraten durch Binden an DnaK fördern. Der *cis*-Effekt von DnaJ auf DnaK in ternären (ATP·DnaK)·Protein·DnaJ-Komplexen (Han and Christen, 2003, 2004) beschleunigt die ATP Hydrolyse und wirkt als Sortierungssystem. Es liest unter den DnaK-gebundenen Proteinsubstraten diejenigen aus, welche im DnaK-Zyklus zu bearbeiten sind; bei 45°C ist der Anteil an Proteinsubstrat, welcher in den Zyklus

geschleust wird, über 20mal höher als derjenige von Peptidsubstrat. Die Thermosensorfunktion von GrpE verzögert die Freisetzung des Proteinsubstrats aus dem Zyklus bei höheren Temperaturen; der Anteil an DnaK im ADP-ligandierten, hochaffinen R-Zustand ist zweimal höher bei 45°C als bei 25°C. Zudem stellen wir fluoreszenzmarkiertes DnaK H541C als taugliches Hilfsmittel vor, um die Assoziation und Dissoziation von DnaK und Proteinsubstraten in Echtzeit zu verfolgen.

General introduction

Protein folding *in vitro* and *in vivo*

In vitro protein folding experiments, in which a protein is unfolded in a solution of denaturant, which is then removed or diluted, have demonstrated that a denatured protein can spontaneously refold into its native structure in the absence of any helper proteins or other cofactors. All the information required to determine the final three-dimensional structure of a protein resides in the polypeptide chain itself (Anfinsen et al., 1961; Anfinsen, 1973). The driving forces for the folding of a polypeptide chain are the same interactions that are responsible for the stabilization of its native conformation: hydrophobic interactions, hydrogen bonds, Coulomb and van der Waals forces (for a concise review, see Fersht, 1999). The native state of a protein corresponds to the thermodynamically most stable conformation that is kinetically accessible under physiological conditions. However, the number of all possible conformations of a polypeptide chain is so large that it would take an astronomical length of time to find the native structure by a systematic search of the total conformational space ("Levinthal paradox"; Levinthal, 1968). Rather, folding of a protein must proceed along a limited number of pathways in order to attain its native structure in a finite time (Wolynes et al., 1995; Dill and Chan, 1997; Karplus, 1997). Rapid formation of compact folding intermediates on a millisecond time scale, initiated by a collapse of the hydrophobic regions into the interior of the molecule, appears to restrict the conformational space available for the polypeptide chain (Fersht, 1999; Dobson, 2004; Zhou et al., 2004).

Folding experiments *in vitro* are usually performed with small, single-domain proteins under conditions, which minimize off-pathway reactions, such as very dilute protein solutions and low temperature. In contrast to these *in vitro* conditions, the interior of a cell is a highly crowded environment with an estimated cytosolic protein concentration as high as 300 mg/ml

(Zimmerman and Trach, 1991). Moreover, during translation, the folding information encoded in the amino acid sequence becomes available in a vectorial fashion, allowing the NH_2-terminal portion of the nascent chain to fold as soon as it has emerged from the ribosome. Productive folding, however, requires that a complete folding domain is available (Jaenicke, 1991). In the cell, off-pathway reactions are reduced by folding catalysts and molecular chaperones, which catalyze specific folding steps and prevent misfolding and aggregation of nascent and denatured proteins.

Molecular chaperones

The proper folding of cellular proteins is mainly controlled by molecular chaperones, which are abundant proteins found in all living organisms (the two major chaperone systems DnaK/DnaJ/GrpE and GroEL/GroES of *E. coli* constitute 15 – 20% of total cellular protein at 46°C (Arsène et al., 2000)). By definition, molecular chaperones do not catalyze folding reactions, but rather increase the number of protein molecules that are on a productive folding pathway (Ellis, 2000). They participate in a variety of cellular processes, including folding of newly synthesized proteins, refolding of misfolded proteins, translocation of proteins across membranes, assembly and disassembly of protein complexes, proteolysis of unstable proteins, and activity control of regulatory proteins (for a recent review, see Mayer and Bukau, 2005). Apparently, all these activities rely on transient interactions of the chaperones with short hydrophobic segments of their protein substrates. The hydrophobic segments typically reside in the interior of folded proteins and are only exposed in proteins that need assistance by molecular chaperones.

Different chaperones employ different strategies for exerting chaperoning action on substrates, which are characteristic of the individual chaperone families (for a synopsis, see Table I).

Table I Conserved families of molecular chaperones[1]

Chaperone family	Prokaryotic Members	Eukaryotic Members	Functions
Hsp100	ClpA, ClpB, ClpX, ClpY	Hsp104, Hsp78	Assistance in proteolysis of unstable proteins; prevention of aggregation of misfolded proteins; disaggregation of misfolded proteins
Hsp90	HtpG	Hsp90, Grp94, ERp99, endoplasmin, Hsp108, gp96	Prevention of aggregation and assistance in refolding of misfolded proteins; regulation of activity of kinases and steroid hormone receptors
Hsp70	DnaK, HscA (Hsc66)	Hsp70, Hsc70, Ssa1-4, Ssb1,2, Ssc, Ssh1, Lhs1, Kar2, BiP, Grp78	Prevention of aggregation and assistance in (re)folding of misfolded proteins and nascent polypeptide chains; activity control of regulatory proteins; translocation of precursors across membranes
Hsp40[2]	DnaJ, DjlA, CbpA, HscB	Hsp40, Ydj1, Sec63, Auxilin, CSPs, Mdj1, Hdj1, Hdj2	Co-chaperone of Hsp70
GrpE	GrpE	Mge1p	Co-chaperone of Hsp70
sHsp	IbpA, IbpB	Hsp18.1, Hsp25, Hsp27, αβ-crystallin	Prevention of aggregation and assistance in refolding of misfolded proteins
Chaperonin	GroEL	Hsp60, Cpn60	Prevention of aggregation and assistance in (re)folding of misfolded and newly synthesized proteins
	GroES	Hsp10, Cpn10	Co-chaperone of GroEL
Calnexin	—	Calnexin	Folding of proteins in the ER
	—	Calreticulin	Folding of proteins in the ER

[1]Table adapted from Bukau et al., 1999; only selected chaperone families and members thereof are shown.
[2]Hsp40 proteins share the J domain, a conserved fragment of about 70 amino acid residues, which is essential for interaction with Hsp70 proteins.

ClpB, a member of the 100-kDa heat shock protein (Hsp100) family, forms a two-tiered hexameric ring (Lee et al., 2003) with a substrate-binding site localized at the central pore (Schlieker et al., 2004). For disaggregation of protein aggregates, the substrate polypeptide

chain is translocated through the central pore of the chaperone (Weibezahn et al., 2004). Hsp90 form a dimeric clamp; a protein substrate is presumed to bind within the clamp, contacting several hydrophobic binding surfaces in its jaws (Harris et al., 2004). Hsp70 apparently are active as monomers and contain a single substrate-binding site (Zhu et al., 1996; Landry et al., 1992). They assist the folding of nascent and denatured proteins presumably by "passively" shielding exposed hydrophobic stretches as well as by "actively" performing conformational work on misfolded segments of their substrates (Pierpaoli et al., 1997; Mayer et al., 2000; Slepenkov and Witt, 2002; Ben-Zvi et al., 2004). Chaperonins (Hsp60) are large cylindrical protein complexes composed of two stacked rings of seven to nine subunits each (Braig et al., 1994). The apical domains of each subunit contain a patch of hydrophobic amino acids, which face the interior of the cavity and bind unfolded protein substrates through hydrophobic contacts (Fenton et al., 1994). It has been suggested that the enclosed cavity functions as an "Anfinsen cage", i.e. a protected chamber that isolates the polypeptide under conditions of infinite dilution and allows it to fold according to its thermodynamic potential (Ellis, 2003). Moreover, a polypeptide chain interacts simultaneously with several binding sites in the interior of the cage (Zahn et al., 1994; van der Vaart et al., 2004); this feature is most likely a key property of chaperonins allowing partial unfolding of protein substrates. Small heat shock proteins (sHsp) form oligomers with an average size of 12 to 42 subunits (Kim et al., 1998). Each oligomer can bind several protein substrates, up to one molecule per subunit; sHsp thus qualify as very efficient binding scaffolds for misfolded proteins.

Differences between the chaperone families also exist with regard to activity control. The substrate binding and release cycle of ClpB/Hsp100, Hsp90, Hsp70 and the chaperonins is controlled by ATP. The energy of ATP drives conformational changes in the substrate-binding site of the chaperones which alter its affinity for substrate and control substrate binding and release (for a review, see Frydman, 2001). The ATPase activity of these

chaperones is a prime target for regulatory proteins which either stimulate or retard the ATPase cycle. Some chaperones, including sHsp, act independently of ATP. In this case, however, binding and release of protein substrates is rather slow. Apparently, sHsp cooperate with the ATP-dependent Hsp70 or ClpB/Hsp100 systems for efficient chaperoning action (Mogk et al., 2003; Haslbeck et al., 2005).

The DnaK/DnaJ/GrpE chaperone system of *E. coli*

DnaK – DnaK is a member of the Hsp70 family. It performs its chaperone activity in the cytoplasm of *E. coli*, where it assists the folding and assembly of newly synthesized proteins, refolding of misfolded and aggregated proteins, translocation of secretory proteins and the control of the activity of regulatory proteins (for a recent review, see Mayer and Bukau, 2005). DnaK consists of an NH_2-terminal ATPase domain of 44 kDa and a COOH-terminal substrate-binding domain of 25 kDa (Figure 1). Binding and hydrolysis of ATP induce conformational changes in the ATPase domain, which are communicated (Jiang et al., 2005) to the substrate-binding domain and modulate its affinity for substrates. In the ADP-liganded R state, DnaK exhibits high affinity for substrates and slow rates of binding and release (Palleros et al., 1993; Schmid et al., 1994), the dissociation equilibrium constants of (ADP·DnaK)·peptide complexes being in the range of 50 nM to 2 μM (Pierpaoli et al., 1998). The ATP-liganded T state is characterized by low substrate affinity (dissociation equilibrium constants of T-state DnaK are about two orders of magnitude higher than that of R-state DnaK (Pierpaoli et al., 1998)) and fast kinetics, allowing rapid binding and release of substrate. Screening of peptide libraries for DnaK-binding sites (Gragerov et al., 1994; Rüdiger et al., 1997) revealed that DnaK recognizes preferentially peptide segments composed of a hydrophobic core of four to five apolar residues, in particular leucine, but also isoleucine, valine, phenylalanine or tyrosine residues, and flanking regions with basic residues.

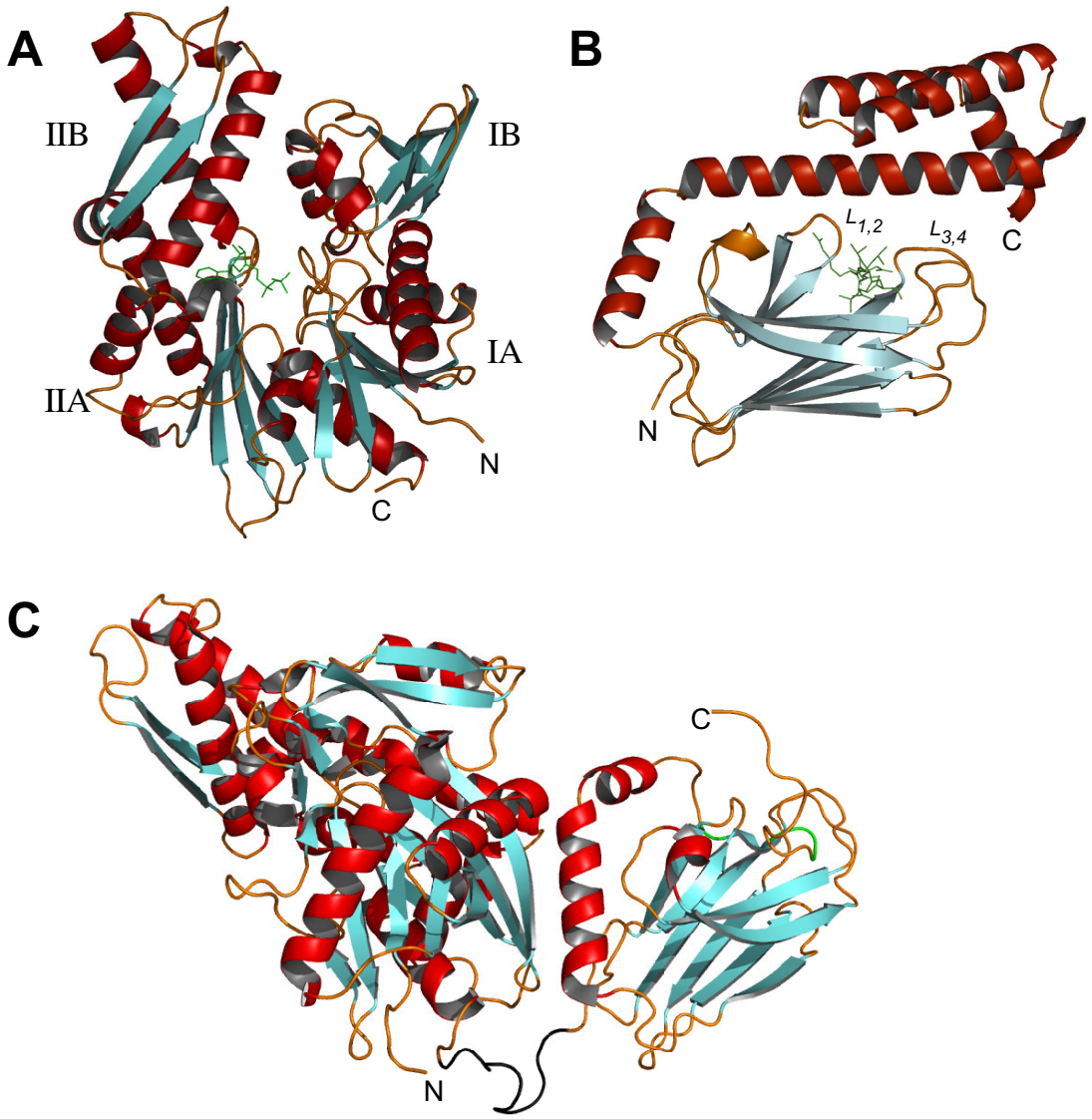

Figure 1 Crystal structures of the bovine Hsc70 ATPase domain, the substrate-binding domain of DnaK and the overall structure of bovine Hsc70. (**A**) The ATPase domain of Hsc70 (residues 3-384) comprises two large globular lobes (I and II), which are separated by a deep central cleft and connected by two crossed α-helices. Each lobe can be divided into a lower subdomain A and an upper subdomain B. In the active site, located at the bottom of the central cleft, two phosphate-binding loops and a hydrophobic adenosine-binding pocket interact with ADP (depicted by green sticks). The precise geometry of the nucleotide and the surrounding residues requires the correct positioning of a Mg^{2+} ion, enabled in part by the binding of two K^+ ions nearby (not shown). The structure of the ATPase domain of DnaK is almost identical to that of bovine Hsc70. The atomic coordinates are from Protein data bank entry 3HSC (Flaherty et al., 1990). (**B**) The substrate-binding domain of DnaK (residues 393-607, Δ608-638) forms a compact β-sandwich, composed of two sheets of four antiparallel strands each, which is followed by an extended structure of α-helices spanning back over the β-sandwich. The substrate-binding site is primarily formed by the two loops $L_{1,2}$ and $L_{3,4}$ of the β-sandwich subdomain. The substrate peptide NR (NRLLLTG) (depicted by green sticks) is unidirectionally bound in extended conformation (see also Landry et al., 1992; Feifel et al., 1998; Rüdiger et al., 2001) and interacts with DnaK through numerous van der Waals contacts of its main chain and side chains as well as seven main-chain hydrogen bonds. The α-helical segments do not directly participate in the binding of the peptide; they appear to serve as a flexible lid over the substrate-binding cavity.

Binding sites for DnaK on polypeptide chains occur on average every 36 residues irrespective of the type of protein (Rüdiger et al., 1997). Thus, several DnaK molecules may bind to one polypeptide chain. DnaK possesses only a weak intrinsic ATPase activity (Zylicz et al., 1983). To perform its chaperone activity on substrates, DnaK acts together with the co-chaperones DnaJ and GrpE, which conjointly accelerate the ATPase activity of DnaK (Liberek et al., 1991; Packschies et al., 1997; Laufen et al., 1999).

DnaJ – DnaJ is a member of the Hsp40 family. The functional active form of DnaJ has been proposed to be a dimer (Zylicz et al., 1985); however, monomers and oligomers up to 20-mers are found to occur *in vitro* (Schönfeld et al., 1995a). DnaJ is a multidomain protein composed of an NH_2-terminal, highly conserved J domain, a Gly/Phe-rich region, a zinc-binding domain and a COOH-terminal low-homology region (Figure 2). The J domain, together with the Gly/Phe-rich region, interacts with DnaK and efficiently stimulates the hydrolysis of DnaK-bound ATP (Wall et al., 1995; Karzai and McMacken, 1996). DnaJ not only stimulates the ATPase activity of DnaK, but also associates with unfolded proteins and prevents protein aggregation (Langer et al., 1992; Gamer et al., 1996; Rüdiger et al., 2001). Binding of substrates to DnaJ requires the zinc-binding domain and the COOH-terminal region (Szabo et al., 1996). DnaJ recognizes similar amino acid sequences as DnaK, preferring clusters of hydrophobic and, in contrast to DnaK, especially aromatic residues.

The lid is supposed to be predominantly in an either opened or closed conformation in ATP-liganded and ADP-liganded (or nucleotide-free) DnaK, respectively. The atomic coordinates are from Protein data bank entry 1DKX (Zhu et al., 1996). (**C**) The NH_2-terminal ATPase domain of Hsc70 is connected by an exposed linker (shown in black) to the COOH-terminal substrate-binding domain. The substrate-binding domain is truncated (residues 1-554, Δ555-650), the lid covering the substrate-binding cleft (see the substrate-binding domain of DnaK in Panel B) is absent. Helix 1 of the substrate-binding domain rests in a groove between lobes IA and IB of the ATPase domain. Helix 2 of the substrate-binding domain is partially unwound, and residues 539-544 (shown in green) occupy the substrate-binding site. Apparently, ATP binding causes the exposed linker to intrude into the interdomain interface, which may destabilize the interactions between the lid and the β-sandwich. The atomic coordinates are from Protein data bank entry 1YUW (Jiang et al., 2005).

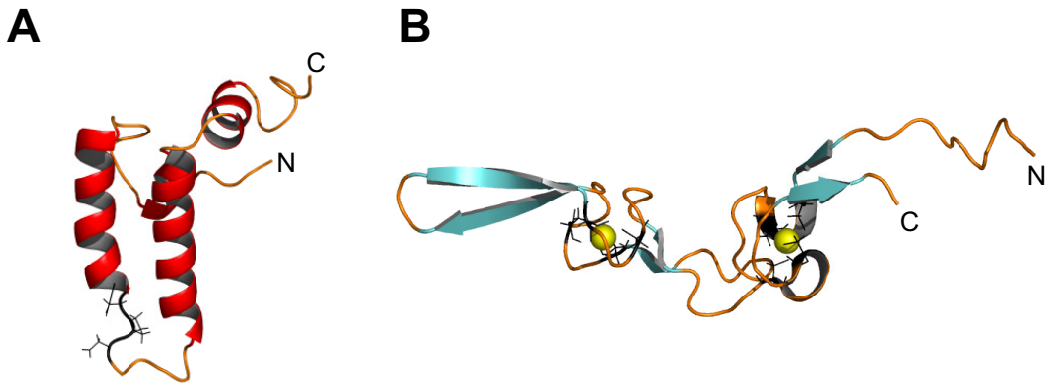

Figure 2 Solution structures of the J domain and the zinc-binding domain of DnaJ. (**A**) The J domain (residues 1-75) consists of four helices, of which the amphipathic antiparallel helices 2 and 3 form a coiled coil connected by a flexible loop, and the helices 1 and 4 are oriented perpendicular to it. The flexible loop contains the entirely conserved sequence motif His33-Pro34-Asp35 (Bork et al., 1992), referred to as HPD motif, which plays a key role in the interaction of DnaJ with DnaK. The Gly/Phe-rich region, located adjacent to the J domain, may serve as flexible linker connecting the J domain with the zinc-binding domain. The conserved HPD motif is depicted by black sticks. The atomic coordinates are from Protein data bank entry 1XBL (Pellecchia et al., 1996). (**B**) The zinc-binding domain (residues 130-208) forms an extended V-shaped structure with two separate zinc-binding modules, one in each wing of the domain. A zinc-binding module comprises two repeats of the sequence motif C-X-X-C-X-G-X-G (X denoting a charged or polar amino acid) with a zinc atom tetrahedrally coordinated to the four cysteine residues (Martinez-Yamout et al., 2000); the structure of the zinc-binding module resembling zinc finger-like domains of certain DNA binding proteins (Szabo et al., 1996). The pronounced groove within the base of the "V" represents a potential binding site for protein substrates. The zinc atoms are represented by yellow dots, and the cysteine residues are depicted by black sticks. The atomic coordinates are from Protein data bank entry 1EXK (Martinez-Yamout et al., 2000).

The binding affinity of DnaJ for substrates is similar to that of DnaK in its low-affinity ATP-liganded state, dissociation equilibrium constants of DnaJ·peptide complexes being in the range from 0.3 µM to 37 µM (Feifel et al., 1998; Pierpaoli et al., 1998). Protein substrates such as firefly luciferase, σ^{32} (Laufen et al., 1999), denatured rhodanese or RepA (Han and Christen, 2003, 2004) accelerate the DnaJ-stimulated hydrolysis of DnaK-bound ATP by one to two orders of magnitude. The acceleration is due to the *cis*-effect of DnaJ on DnaK in ternary (ATP·DnaK)·substrate·DnaJ complexes, the simultaneous binding of DnaK and DnaJ to one and the same polypeptide substrate resulting in a higher effective concentration of DnaJ (Han and Christen, 2003, 2004).

Figure 3 Crystal structure of the GrpE dimer. The NH_2-terminally truncated GrpE dimer (residues 34-197) has been crystallized in complex with the ATPase domain of DnaK (not shown). The two long α-helices lie nearly in the same plane and do not form a classical coiled coil, because the regular i+3, i+4 heptad repeat of hydrophobic residues, which is a prerequisite for forming a canonical coiled coil, is interrupted at two positions. The long helices are connected by two rather disordered loops (not resolved in the crystal structure) to the two short helices of the four-helix bundle. The compact β-sheet domain consists of six short β-strands. The major areas of contact between GrpE and DnaK are the proximal β-sheet domain and the COOH-terminal part of the proximal long α-helix as well as the subdomains IB and IIB in the ATPase domain of DnaK. The structure shown here is that of GrpE(G122D), which has been found to be virtually inactive as an ADP/ATP exchange factor (Grimshaw et al., 2005), but is the only GrpE structure available to date. The atomic coordinates are from Protein data bank entry 1DKG (Harrison et al., 1997).

GrpE – The 22-kDa GrpE protein acts as nucleotide exchange factor for DnaK. The functional active form of GrpE is a homodimer. The GrpE dimer encompasses two NH_2-terminal unstructured tail regions of 33 residues, one in each monomer, a pair of long α-helices, a small four-helix bundle, to which each monomer contributes two helices, and two small β-sheet domains at the COOH-terminus (Figure 3). Dimeric GrpE stably interacts with DnaK in the absence of ATP (Schönfeld et al., 1995b) with a dissociation equilibrium constant of 200 nM for the (ADP·DnaK)·GrpE complex (Packschies et al., 1997), and dissociates from DnaK upon addition of ATP (Zylicz et al., 1987). In the complex, only the

GrpE subunit proximal to DnaK directly interacts with DnaK. The structure of GrpE is strikingly asymmetric, the GrpE dimer being bent toward DnaK. On the basis of conformational differences between the crystal structure of the ATPase domain of DnaK in complex with GrpE (Harrison et al., 1997) and the structure of the Hsc70 ATPase domain without nucleotide exchange factor (Flaherty et al., 1990; Jiang et al., 2005), binding of GrpE has been suggested to induce an opening of the nucleotide-binding cleft of DnaK which facilitates the release of ADP plus inorganic phosphate from DnaK (Harrison et al., 1997). However, any detailed mechanism derived from the structure of the DnaK·GrpE complex (Harrison et al., 1997) remains speculative, because a mutant form of GrpE (GrpE(G122D)) was used for crystallization, which has been found to be virtually inactive as nucleotide exchange factor (Grimshaw et al., 2005).

The DnaK/DnaJ/GrpE chaperone cycle – The intrinsic ATPase activity of DnaK is very low (Zylicz et al., 1983). The rate constants of ATP hydrolysis and of ADP release at 25°C have been found to be 0.0015 s^{-1} and 0.035 s^{-1}, respectively (McCarty et al., 1995; Theyssen et al., 1996). In the cell, the low intrinsic ATPase activity of DnaK is accelerated by up to 3 orders of magnitude in the presence of the co-chaperones DnaJ and GrpE, which stimulate the hydrolysis of DnaK-bound ATP and facilitate the exchange of ADP for ATP, respectively (Packschies et al., 1997; Laufen et al., 1999). The current model of the functional cycle of the DnaK system (Figure 4) can be described as follows: DnaK alternates between two states. The ATP-liganded T state exhibits low affinity for substrates and fast rates of binding and release, whereas the ADP-liganded R state is characterized by high substrate affinity and slow kinetics (Palleros et al., 1993; Schmid et al., 1994). The association of substrate with ADP-liganded R-state DnaK is too slow to be of physiological relevance.

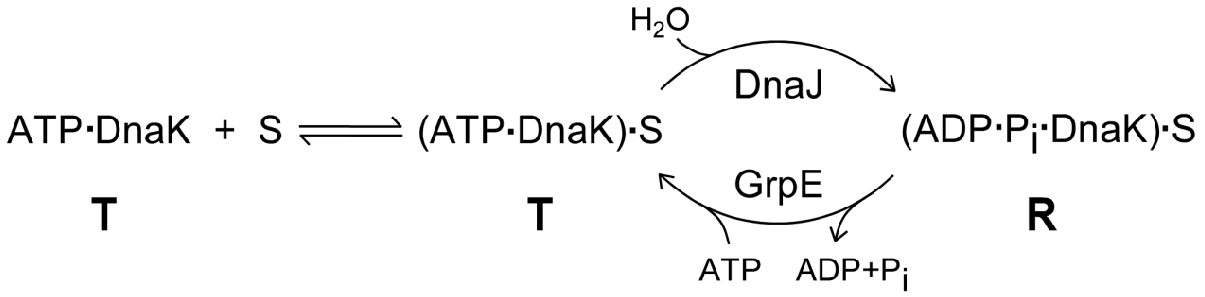

Figure 4 The DnaK/DnaJ/GrpE chaperone cycle. The substrate S first associates with ATP-liganded T-state DnaK. DnaJ then feeds the substrate into the cycle by stimulating the hydrolysis of DnaK-bound ATP (Karzai and McMacken, 1996; Laufen et al., 1999; Han and Christen, 2003). In the high-affinity R state, the substrate remains bound to DnaK (Schmid et al., 1994). GrpE releases the substrate from the cycle by facilitating ADP/ATP exchange (Packschies et al., 1997; Brehmer et al., 2004). The fraction of R-state DnaK, and thus of sequestered substrate, is controlled by the conjoint action of the co-chaperones DnaJ and GrpE.

The ATP-liganded T state allows for rapid association of substrate with DnaK but precludes the formation of stable DnaK·substrate complexes. A substrate thus enters the cycle by fast binding to low-affinity T-state DnaK. DnaJ-stimulated hydrolysis of DnaK-bound ATP promotes the formation of stable (ADP·DnaK)·substrate complexes. GrpE-facilitated ADP/ATP exchange converts DnaK into the low-affnity T state. The protein substrate is either released from T-state DnaK and proceeds along its folding pathway, or it enters again the DnaK chaperone cycle. The formation of high-affinity (ADP·DnaK)·substrate complexes decreases the concentration of free non-native proteins and thus prevents aggregation, whereas the release of protein substrate from the cycle into to solvent allows for folding. Sequestering and release of substrate are tightly controlled by DnaJ and GrpE; the balance of their activities is essential for efficient chaperoning action of DnaK.

The heat shock response

Discovery of the heat shock response – In the early 1960s, Ferrucio Ritossa at the International Laboratory of Genetics and Biophysics in Naples was studying the puffing pattern of the giant chromosomes of *Drosophila* salivary glands (puffs indicate transcribed genes). One day, he observed a new puffing pattern: some chromosomal puffs were induced,

while other preexisting puffs were reduced (Ritossa, 1962). It turned out that one of his colleagues had accidentally shifted the temperature of the incubator. Ritossa explored the puffing pattern under defined conditions and observed an onset of synthesis of new RNA just 2 to 3 min after the temperature upshift. Twelve years later, Alfred Tissières in Geneva found that the protein pattern of *Drosophila* changed after a heat shock from 25°C to 37.5°C (Tissières et al., 1974). By autoradiography of the radioactively labeled proteins separated by gel electrophoresis, he discovered that several protein bands were drastically increased in their intensity, while other bands remained unchanged or disappeared.

Further studies over the past decades revealed that the heat shock response occurs in all three kingdoms, in bacteria, archaea and eukarya. The so-called heat shock proteins turned out to be constitutive cellular components that are also essential for the viability of the cells under non-stress conditions. The heat shock response is also elicited by cellular stress other than heat shock, such as changes in osmolarity and pH, heavy metals, ethanol, free-radical damage and viral infections (for recent reviews, see Schumann, 2000; Wick and Egli, 2004). The common characteristic of these adverse conditions is that they lead to partially unfolded and aggregation-prone proteins. Most heat shock proteins have been found to be either molecular chaperones or proteases, which assist protein folding or degrade misfolded proteins, respectively.

The heat shock response in *E. coli* – *E. coli* cells can grow over a temperature range of almost 40°C. Between 20°C and 37°C, the rate of growth varies as an exponential function of temperature; this range is regarded as the normal growth range (Lemaux et al., 1978; Herendeen et al., 1979).

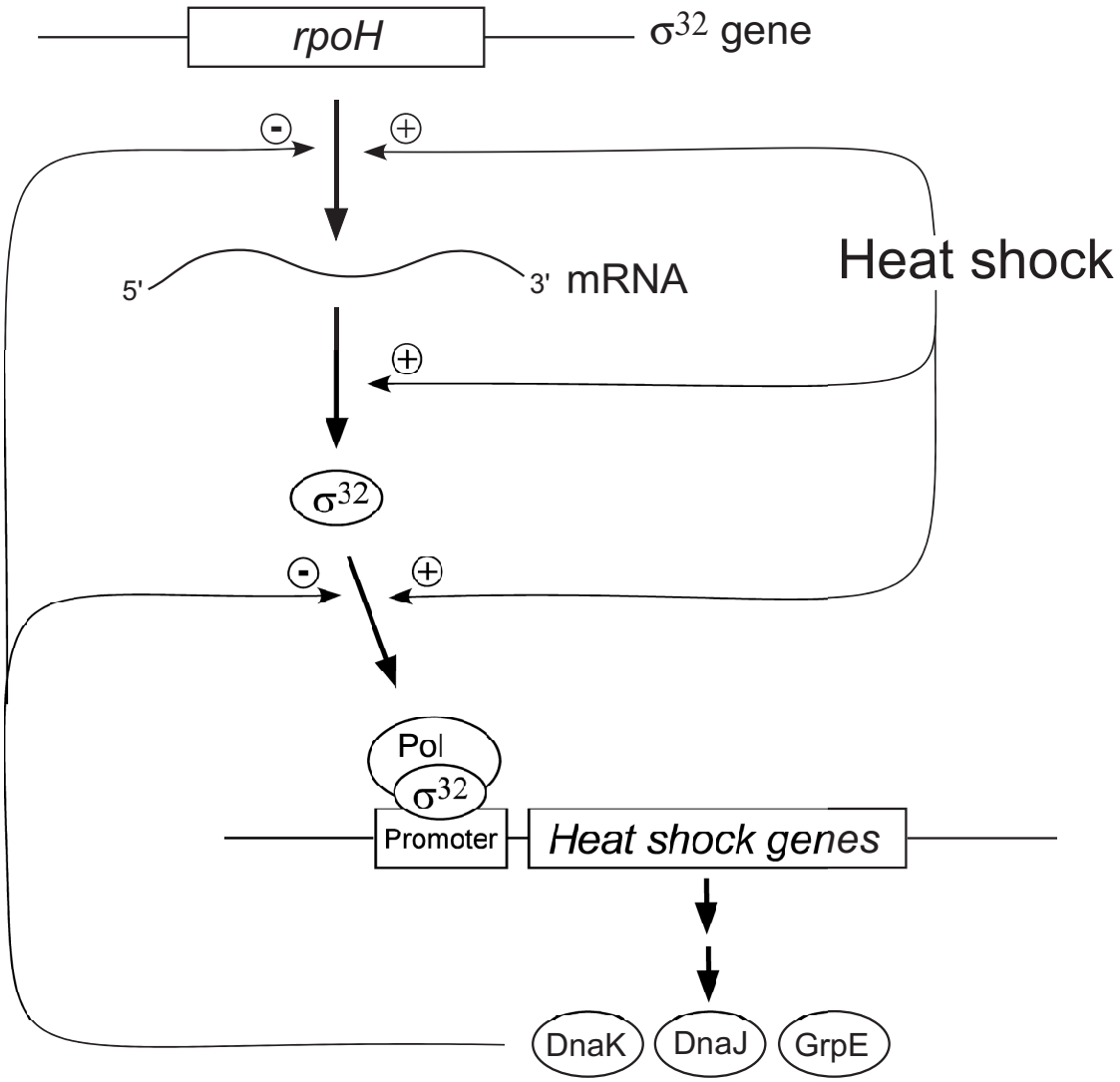

Figure 5 The heat shock response in *E. coli.* Under normal growth conditions, cellular levels of σ^{32} are low due to DNA supercoiling of the *rpoH* gene encoding σ^{32}, the secondary structure of σ^{32} mRNA and rapid degradation of σ^{32} by proteases. Heat shock leads to denaturation of cellular proteins and subsequent binding of these proteins to DnaK. Thereby σ^{32} is competed away from DnaK and interacts with RNA polymerase, enhancing the transcription of heat shock genes. A further increase in σ^{32} levels is due to a decrease in supercoiling of DNA and melting of the mRNA secondary structure. The negative feedback control of σ^{32} by its own transcription/translation product DnaK allows a fast control and fine tuning of the heat shock response (for details, see text). The schematic picture is adapted from Connolly et al., 1999; Wick and Egli, 2004.

The synthesis of heat shock proteins, such as the molecular chaperones ClpB, DnaK, DnaJ, GrpE, GroEL and GroES as well as the proteases FtsH, HslUV and Lon, is controlled at the transcriptional level by the sigma factor 32 (σ^{32}), which as an alternative subunit of RNA polymerase directs the polymerase to promoter sequences upstream of the heat shock genes (for a recent review, see Wick and Egli, 2004). Besides the heat shock factor σ^{32}, there are

several other sigma factors, such as the principal, housekeeping factor σ^{70}, the nitrogen-limitation factor σ^{54} and the stationary phase factor σ^{38}, each of which allow to express specifically a distinct set of proteins. At a temperature of 30°C, there are less than 50 molecules of σ^{32} per cell compared to about 3000 molecules per cell of housekeeping σ^{70} (Craig and Gross, 1991). Upon heat shock from 30°C to 42°C, the cellular concentration of σ^{32} increases about 15-fold, which commensurately enhances the rate of synthesis of heat shock proteins within 5 min (Neidhardt and VanBogelen, 1987; Straus et al., 1987). After reaching the steady state at 42°C, the cellular concentrations of DnaK, DnaJ and GrpE are about 2-fold greater than at 30°C (Mogk et al., 1999; Liu, 2002). σ^{32} is regulated on the transcriptional, the translational and the posttranslational level (Figure 5). DNA supercoiling has been suggested to be reduced at heat shock temperatures, which enhances transcription of the *rpoH* gene encoding σ^{32} (Lopez-Garcia and Forterre, 2000). The changes in DNA topology are directly induced by elevated temperatures as well as by thermal modulation of the activities of topoisomerases. DnaK facilitates the recovery of the supercoiled DNA topology by stimulating the activity of topoisomerase gyrase (Ogata et al., 1996). The mRNA of σ^{32} acts as thermosensor (Morita et al., 1999a); at normal growth temperature, the *rpoH* mRNA adopts a secondary structure that masks both the ribosome-binding site and the start codon, impeding efficient translation (Morita et al., 1999b). At elevated temperatures, this secondary structure melts, leading to ribosome binding and increased σ^{32} synthesis. The stability of σ^{32} is dependent on the amount of misfolded proteins in the cell (Bukau, 1993; Tomoyasu et al., 1998). Under normal growth conditions, most of σ^{32} is bound by DnaK and subsequently degraded by proteases within less than 1 min. Upon heat shock, the increasing amount of misfolded proteins competes σ^{32} off DnaK, resulting in binding of σ^{32} to RNA polymerase and increased expression of heat shock proteins. DnaK and the proteases, which

are regulated themselves by σ^{32}, thus exert a negative feedback control of the heat shock response.

Temperature dependence of structure and activity of DnaK, DnaJ and GrpE

Molecular chaperons are both essential for growth under normal conditions and for surviving heat shock temperatures, at which many proteins are heat-denatured and prone to aggregation. Chaperones have been assumed to be stable at elevated temperatures. DnaK and DnaJ indeed have proven to be heat-stable; no major conformational changes have been observed between 15°C and 48°C (Grimshaw et al., 2001). However, GrpE undergoes a conformational transition within the physiologically relevant temperature range, as observed with circular dichroism spectroscopy and differential scanning calorimetry (Grimshaw et al., 2001; Gelinas et al., 2002). The temperature-induced conformational transition in GrpE is fully reversible. The transition initiates around 35°C and reaches its midpoint at ~48°C. The transition midpoint is independent of the GrpE concentration in the range from 0.1 μM to 290 μM (Grimshaw et al., 2001; Gelinas et al., 2002), indicating that the transition does not include changes in the monomer/dimer equilibrium of GrpE. Consistent with the structural data, the rate of the DnaJ-stimulated T→R conversion exhibits an Arrhenius temperature dependence between 15°C and 48°C, whereas the rate of the GrpE-facilitated R→T conversion increases less and less with increasing temperature and even decreases above ~40°C (Grimshaw et al., 2001). The thermosensor function has been attributed to the pair of long α-helices in the GrpE dimer (Grimshaw et al., 2003; Gelinas et al., 2003). An engineered disulfide bond (R40C) in the long helix pair stabilized GrpE against thermal unfolding (Grimshaw et al., 2003). The thermal transition observed with wt GrpE was absent in the disulfide-stabilized GrpE(R40C), and the rate of the GrpE(R40C)-facilitated R→T conversion continuously increased with

increasing temperature from 15°C to 48°C. Apparently, GrpE acts as a thermosensor adapting the DnaK/DnaJ/GrpE chaperone system to heat shock conditions. At elevated temperatures, the reversible thermal transition in GrpE may be expected to shift DnaK toward the high-affinity R state, favoring sequestration of substrate by DnaK.

Aim of this work

In this study we investigate the concept of a direct heat shock response of the DnaK/DnaJ/GrpE chaperone system, that adapts the DnaK cycle instantly and reversibly to heat shock conditions, DnaJ acting as non-native-protein sensor (Siegenthaler and Christen, 2006) and GrpE as thermosensor (Grimshaw et al., 2001, 2003; Groemping and Reinstein, 2001; Gelinas et al., 2002, 2003; Moro and Muga, 2006). The following questions were tackled:

(1) Is DnaK shifted toward its high-affinity R state by the conformational transition in GrpE at elevated temperatures and does it consequently sequester a higher fraction of substrate?

(2) Is the thermosensor function of GrpE advantageous for the DnaK chaperone action during heat shock, i.e. for protecting protein substrates against heat denaturation and aggregation?

(3) What is the functional role of the *cis*-effect of DnaJ on DnaK in ternary (ATP·DnaK)·substrate·DnaJ complexes in the chaperone cycle? A major question in the chaperone field is how targeted chaperone action is selectively triggered. Protein substrates accelerate the DnaJ-stimulated ATP hydrolysis by one to two orders of magnitude (Laufen et al., 1999; Han and Christen, 2003, 2004). DnaJ not only stimulates the ATPase activity of DnaK, but also associates with unfolded proteins and prevents protein aggregation (Langer et al., 1992; Gamer et al., 1996; Rüdiger et al., 2001). DnaJ has been suggested to select the chaperone substrates and then to transfer them to DnaK (Szabo et al., 1994; Gamer et al.,

1996; Laufen et al., 1999; Rüdiger et al., 2001). However, no experimental evidence has been reported as yet for this substrate transfer. A plausible mechanism of the targeting action of DnaJ is provided by the concept of a *cis*-effect of DnaJ on DnaK in ternary (ATP·DnaK)·protein·DnaJ complexes (Han and Christen, 2003, 2004): the simultaneous binding of DnaK and DnaJ to one and the same polypeptide chain results in a higher effective concentration of DnaJ.

(4) How much do DnaJ as non-native-protein sensor and GrpE as thermosensor contribute to the dynamic sequestering of substrate by DnaK under heat shock conditions? Is there a simple means to follow the interaction of DnaK with protein substrates in real time? Does DnaK bind and release protein substrates with similar rates as short peptide substrates? Fluorescence-labeled peptides have allowed kinetic analysis of complex formation with DnaK (Schmid et al., 1994; Witt and Slepenkov, 1999). However, no kinetic data of complex formation of DnaK and proteins, the physiological substrates, have been reported to date; the interaction between DnaK and protein substrates having solely been investigated by size-exclusion chromatography (Palleros et al., 1993), non-denaturing gel electrophoresis (Harrison et al., 1997) and measuring fluorescence anisotropy under steady-state conditions (Chattopadhyay and Roy, 2002).

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Immediate response of the DnaK molecular chaperone system to heat shock

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Abstract

The familiar heat shock response in cells comprises the enhanced expression of molecular chaperones. In recent experiments with the Hsp70 system of *Escherichia coli*, the co-chaperone GrpE has been found to undergo a reversible thermal transition in the physiological temperature range. Here, we tested whether this thermal transition is of functional significance in the complete DnaK/DnaJ/GrpE chaperone system. We found that a mere increase in temperature resulted in a higher fraction of fluorescence-labeled peptides being sequestered by DnaK. This direct adaptation of the DnaK/DnaJ/GrpE chaperone system to heat shock conditions may serve to bridge the time lag of enhanced chaperone expression.

Introduction

Molecular chaperones of the 70-kDa heat shock protein (Hsp70) family are essential for the cell to survive environmental stress including heat shock (Connolly et al., 1999; Mayer et al., 2001). To prevent protein misfolding and aggregation, the chaperones transiently interact with hydrophobic segments of nascent and denatured proteins in an ATP-driven cycle (McCarty et al., 1995; Theyssen et al., 1996). Binding and hydrolysis of ATP induce conformational changes in the NH_2-terminal ATPase domain (Flaherty et al., 1990; Harrison et al., 1997), which are communicated to the COOH-terminal peptide-binding domain (Zhu et al., 1996) and modulate its affinity for substrates. The ATP-liganded T state is characterized by low affinity for substrates and fast rates of binding and release, whereas the ADP-liganded R state shows high affinity for substrates and slow kinetics (Palleros et al., 1993; Schmid et al., 1994). DnaK, an Hsp70 homolog in *E. coli*, acts in concert with its co-chaperones DnaJ, an Hsp40 homolog, and GrpE (Liberek et al., 1991; McCarty et al., 1995; Pierpaoli et al., 1998). DnaJ stimulates the hydrolysis of DnaK-bound ATP and converts T-state DnaK into high-affinity R-state DnaK (Figure 1).

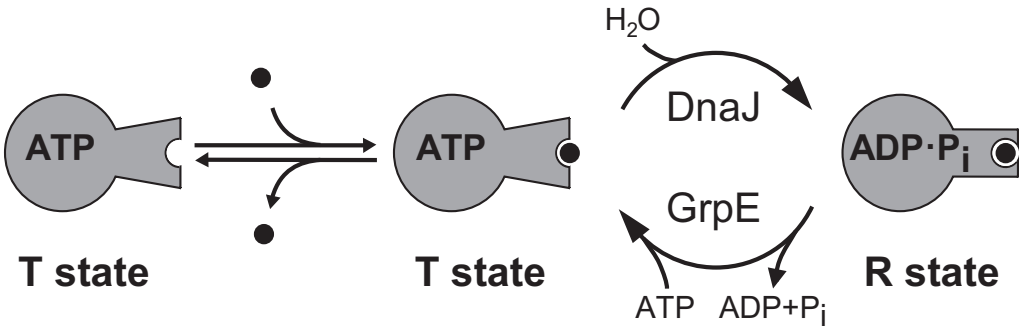

Figure 1 The DnaK/DnaJ/GrpE chaperone cycle. The substrate is fed into the cycle by fast binding to ATP-liganded T-state DnaK (Schmid et al., 1994; McCarty et al., 1995). DnaJ-stimulated hydrolysis of DnaK-bound ATP locks DnaK onto the substrate; direct release of substrate from ADP-liganded R-state DnaK is negligibly slow (Schmid et al., 1994). GrpE-catalyzed ADP/ATP exchange completes the cycle and releases the substrate. The fraction of R-state DnaK, and thus of sequestered substrate, is controlled by the joint action of the co-chaperones DnaJ and GrpE.

GrpE facilitates the ADP/ATP exchange and reconverts the R state to the low-affinity T state. The two co-chaperones DnaJ and GrpE conjointly control the fraction of substrate that is sequestered by high-affinity R-state DnaK. Recently, GrpE has been found to undergo a reversible thermal transition within the physiologically relevant temperature range (Grimshaw et al., 2001; Groemping and Reinstein, 2001; Gelinas et al., 2002), while in DnaK and DnaJ no conformational transitions have been observed in this temperature range (Grimshaw et al., 2001). Consistent with the structural data, the rate of the DnaJ-triggered T→R conversion follows an Arrhenius temperature dependence, whereas the rate of the GrpE-dependent R→T conversion increases less and less with increasing temperature and even decreases above 40°C (Grimshaw et al., 2001). Stabilizing the long NH_2-terminal helix pair of GrpE by an engineered disulfide bond abolishes the thermal transition in GrpE and reduces the deviation of the ADP/ATP exchange activity of GrpE from an Arrhenius temperature dependence, indicating that the long helix pair acts as the primary thermosensor of the chaperone system (Grimshaw et al., 2003; Gelinas et al., 2003). The present study with the complete DnaK/DnaJ/GrpE chaperone system shows that the thermal responsiveness of GrpE is of functional significance. A mere increase in temperature results in a shift of DnaK from its

low-affinity T state toward its high-affinity R state and thus in a higher fraction of substrate being sequestered by DnaK.

Materials and methods

Materials – DnaK was expressed and purified as described previously (Feifel et al., 1996). Its concentration was determined photometrically with ε_{280} = 14,500 $M^{-1}cm^{-1}$ (Hellebust et al., 1990). Purified DnaK contained less than 0.1 mol nucleotide/mol DnaK (Feifel et al., 1996) and was stored at –80°C. DnaJ and GrpE, prepared as reported previously (Schönfeld et al., 1995a; Schönfeld et al., 1995b), were a gift from Dr. H.-J. Schönfeld, Basel. The stock solutions in 50 mM Tris/HCl, 100 mM NaCl, pH 7.7 were kept at –80°C. Peptide NR (NRLLLTG) was purchased from Chiron, Australia (purity >95%) and peptide p4 (CALLQSRLLS) was synthesized by Dr. S. Klauser in our Institute with an ABI 430A Peptide Synthesizer (Applied Biosystems). ATP-Na_2 (purity >98%) and ADP-Na_2 (purity >90%) were purchased from Fluka, Switzerland, and 6-acryloyl-2-dimethylaminonaphthalene (acrylodan) was from Molecular Probes, Eugene, OR.

Labeling of peptides with acrylodan – Peptide p4 is a derivative of the prepiece of mitochondrial aspartate aminotransferase (Schmid et al., 1994); peptide NR is another good DnaK binder (Gragerov et al., 1994) which has been co-crystallized with the substrate-binding domain of DnaK (Zhu et al., 1996). The acryl group of acrylodan served to attach covalently the environmentally sensitive fluorophore to the α-amino group or the sulfhydryl group of peptide NR or p4, respectively. For labeling, NR and p4 were reacted in *N,N*-dimethylformamide with acrylodan at a molar ratio of 1.2 and 0.8, respectively, for 3 h at room temperature. The samples were diluted with water to 10% (v/v) *N,N*-dimethylformamide, filtrated (0.2 μm), and purified by reverse-phase HPLC as reported

previously (Pierpaoli et al., 1997). Molecular mass and purity of the acrylodan-labeled peptides were confirmed by mass spectrometry. The concentrations of the stock solutions in 30% (v/v) acetonitrile were determined photometrically with ε_{380} = 20,000 $M^{-1}cm^{-1}$ (Molecular Probes).

Fluorescence spectra – A Perkin-Elmer spectrofluorimeter LS50B, equipped with a stirrer and a thermostated cuvette holder, was used to record fluorescence emission spectra of a-peptides. The solution was heated and cooled at a rate of ~4°C per min. All experiments were performed in assay buffer (25 mM Hepes/NaOH, 100 mM KCl, 10 mM $MgCl_2$, pH 7.0) in a final volume of 800 µl (1 × 0.4 cm cuvette). Throughout, ADP was added together with inorganic phosphate at the same concentration. The excitation wavelength was set at 370 nm (Raman scattering at 422 nm; bandpass 4 nm) and the spectra were recorded from 400 to 600 nm (bandpass 4 nm) under steady-state conditions.

Determination of dissociation equilibrium constants – The dissociation equilibrium constants (K_d) of the DnaK·a-NR complex in the presence of 1 mM ADP (plus 1 mM inorganic phosphate) or 1 mM ATP were determined by fluorescence titration of peptide a-NR (50 nM) with up to 2.5 µM or up to 35 µM DnaK, respectively. A Spex Fluorolog spectrofluorimeter equipped with a stirrer and a thermostated cuvette holder was used. Titration experiments were performed in assay buffer at a starting volume of 800 µl (1 × 0.4 cm cuvette). The excitation wavelength was set at 370 nm (bandpass 4.6 nm) and emission spectra (bandpass 18.5 nm) were recorded from 400 nm to 600 nm. Fluorescence intensity and DnaK concentration were linearly corrected for the increased sample volume after addition of DnaK. K_d and ΔF_{max} values were determined from a least-squares fit of the data with the equation

$\Delta F = PL \cdot \Delta F_{max}/P_t = [\Delta F_{max}/(2P_t)][(K_d+L_t+P_t) - ((K_d+L_t+P_t)^2 - 4P_t \cdot L_t)^{0.5}]$,

where PL denotes the concentration of DnaK·a-NR complex, P_t the total a-NR concentration, L_t the total concentration of DnaK and ΔF the difference in fluorescence intensity at 500 nm between a-NR in the absence and presence of increasing concentrations of DnaK. Analogous calculations based on the difference in the area between 440 nm and 600 nm gave virtually the same K_d values.

Fast kinetic measurements – A stopped-flow apparatus (Applied Photophysics SX18 MV) served to record changes in fluorescence of the a-peptides upon binding to DnaK. The temperature of the syringes and the cuvette was controlled with a circulating external water bath (± 0.5°C). The excitation wavelength was set at 370 nm (bandpass 4.5 nm) and the emitted light passed through a high-pass filter with a 455 nm cut-off. The solutions were equilibrated for at least 5 min at the respective temperature before starting the reaction by mixing volumes of 70 µl each. All experiments were performed in assay buffer. ADP was added together with inorganic phosphate at the same concentration. Reaction traces of at least 5 measurements were averaged and the reaction rates determined from a double-exponential least-squares fit.

Results and discussion

Calibration of the experimental set-up – The goal of this study was to explore in the complete DnaK/DnaJ/GrpE chaperone system whether an increase in temperature shifts DnaK toward its high-affinity R state. To detect this shift, we used fluorescence-labeled peptides (a-peptides, "a" denoting the fluorescent probe acrylodan, see Materials and methods) as substrates for DnaK.

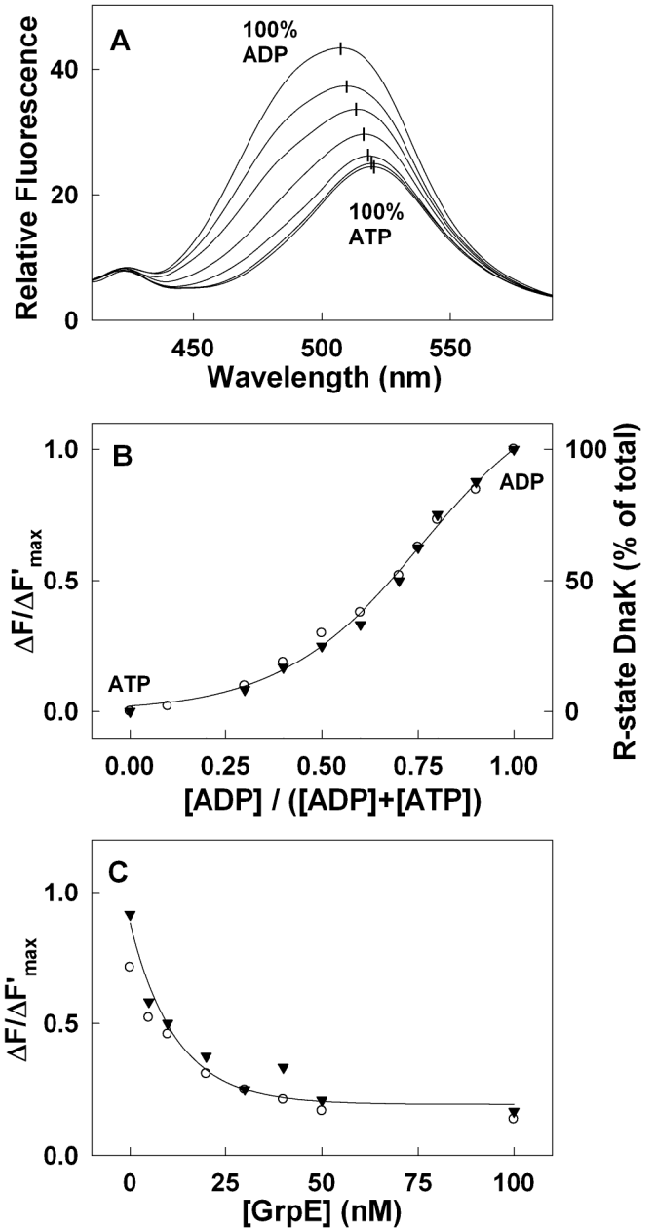

Figure 2 Calibration of experimental set-up. (**A**) Fluorescence emission spectra (λ_{ex} 370 nm) of 200 nM a-NR (final concentrations are indicated throughout), 1 µM DnaK and 1 mM nucleotides (total concentration) were recorded at 25°C at the following [ADP]/([ADP]+[ATP]) ratios: 1 (100% ADP), 0.8, 0.7, 0.5, 0.3, 0.1, 0 (100% ATP). (**B**) $\Delta F/\Delta F'_{max}$ values were calculated on the basis of the fluorescence intensity at 500 nm (○) or λ_{max}^{em} (▼) and plotted *versus* [ADP]/([ADP]+[ATP]). The *solid line* represents the best fit to a rectangular hyperbola. (**C**) Plot of $\Delta F/\Delta F'_{max}$ calculated on the basis of the fluorescence intensity at 500 nm (○) or λ_{max}^{em} (▼) versus [GrpE]. Spectra of 200 nM a-NR, 1 µM DnaK, 200 nM DnaJ, 1 mM ATP and 4 mM ADP at varying GrpE concentrations at 25°C (not shown) served to calculate the $\Delta F/\Delta F'_{max}$ values. The *solid line* represents the best fit to a rectangular hyperbola.

Binding of labeled peptides such as a-NR and a-p4 to DnaK causes both a blue shift of the emission maximum and an increase in fluorescence emission intensity (Schmid et al., 1994) and thus allows to differentiate the two functional states of DnaK according to their affinity

for substrates. Because ADP and ATP compete for the nucleotide-binding site in DnaK, the ADP/ATP concentration ratio determines, in the absence of the co-chaperones, the concentration ratio of ADP-liganded, high-affinity R-state DnaK and ATP-liganded, low-affinity T-state DnaK.

To calibrate the experimental set-up, we measured the extent of DnaK·a-NR complex formation in the absence of the co-chaperones as a function of the nucleotide concentration ratio (Figure 2A). Virtually no peptide was expected to bind to ATP-liganded DnaK at the chosen concentrations, because of the low substrate affinity of T-state DnaK (Table I). The emission spectrum of a-NR in the presence of DnaK and ATP was indeed superimposable with the a-NR spectrum in the absence of DnaK (not shown). Thus, the blue shift of the emission maximum and the increase in fluorescence intensity with increasing ADP concentration reflect binding of a-NR to high-affinity R-state DnaK. The very slow hydrolysis of DnaK-bound ATP does not significantly influence the T state/R state concentration ratio of DnaK because spontaneous ADP/ATP exchange is 10 times faster than hydrolysis (Theyssen et al., 1996).

To estimate the fraction of R-state DnaK, i.e. [R state]/([R state]+[T state]), we used either the wavelength of maximum emission (λ_{max}^{em}) or the fluorescence intensity at 500 nm as measured parameter F in the equation $\Delta F/\Delta F'_{max} = (|F-F_0|)/(|F'_{max}-F_0|)$. Values of F_0 and F'_{max} were obtained from the emission spectra in the presence of ATP or ADP, respectively. Thus, $\Delta F/\Delta F'_{max}$ values of 0 and 1 correspond to T-state and R-state DnaK, respectively. In a plot of $\Delta F/\Delta F'_{max}$ versus [ADP]/([ADP]+[ATP]), very similar results were obtained when either λ_{max}^{em} or the fluorescence intensity at 500 nm was used for calculation (Figure 2B).

For experimentation with the complete DnaK/DnaJ/GrpE chaperone system, the same concentrations of DnaK and peptide were used as above and a DnaJ concentration of 200 nM was chosen according to the physiological DnaK/DnaJ concentration ratio in *E. coli* (Bardwell et al., 1986; Liu, 2002). To determine the concentration range of GrpE in which a change in

its ADP/ATP exchange activity would have an observable effect on the fraction of R-state DnaK, we measured the formation of the DnaK·a-NR complex at 25°C in the presence of DnaJ, ATP and ADP at varying GrpE concentrations. Because GrpE shifts DnaK toward its T state we used an [ADP]/([ADP]+[ATP]) ratio of 0.8 to start the titration with DnaK being predominantly in the R state. $\Delta F/\Delta F'_{max}$, i.e. the fraction of R-state DnaK, decreased with increasing GrpE concentration reaching a minimum at a concentration of ~50 nM (Figure 2C). For further experimentation, a GrpE concentration of 40 nM was chosen. At this concentration, a temperature-dependent decrease in the nucleotide exchange activity of GrpE was expected to result in an observable shift of DnaK toward its R state, a decrease in exchange activity having the same effect as a decrease in GrpE concentration.

Effect of temperature on the fraction of high-affinity R-state DnaK in the complete DnaK/DnaJ/GrpE chaperone system − The effect of temperature on the fraction of R-state DnaK was measured at fixed concentrations of DnaK, DnaJ, GrpE, a-NR, ATP and ADP. Increasing temperature resulted in a progressive blue shift of λ_{max}^{em} (Figure 3A), indicating a shift of DnaK toward its high-affinity R state. The fluorescence intensity decreased with increasing temperature due to enhanced collisional quenching (Pesce et al., 1971). In the control without the co-chaperones (Figure 3B), the thermal quenching of fluorescence was not compensated in part by increased formation of the DnaK·a-NR complex as observed in Figure 3A. In contrast to the fluorescence intensity at 500 nm, λ_{max}^{em} proved to be temperature-independent. The value of $\Delta F/\Delta F'_{max}$ in the presence of the co-chaperones was therefore calculated with λ_{max}^{em} as measured parameter and plotted as a function of temperature (Figure 3C). From the $\Delta F/\Delta F'_{max}$ values we conclude that the fraction of R-state (i.e. peptide-liganded) DnaK increased from ~25% of total DnaK at 15°C to ~75% at 45°C.

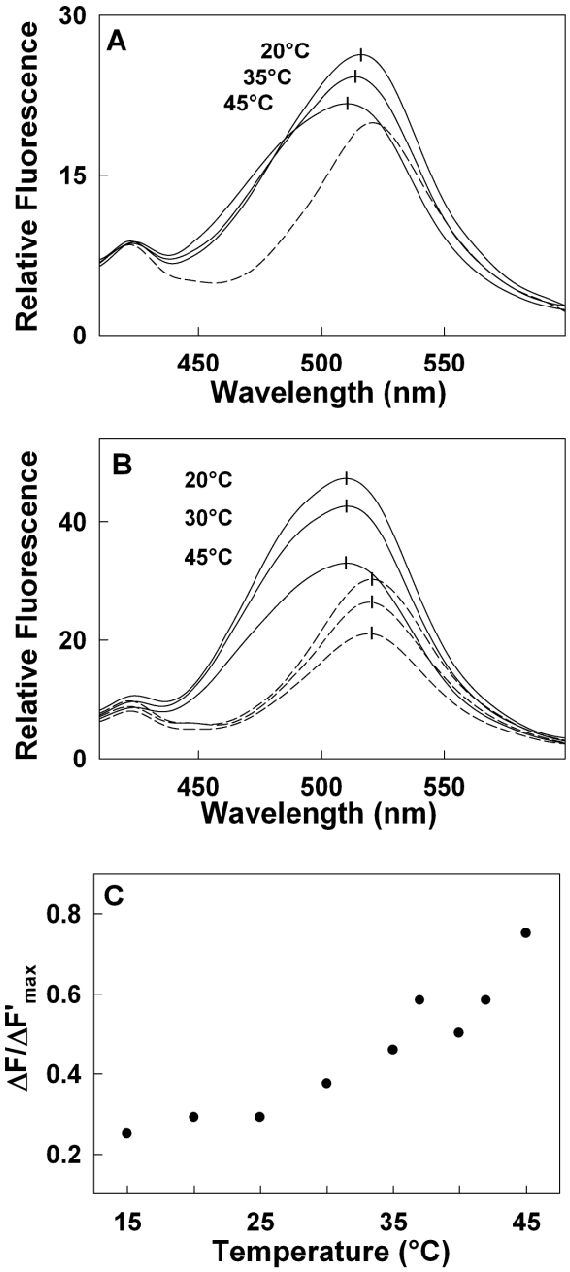

Figure 3 Formation of the DnaK·a-NR complex in the presence of DnaJ and GrpE as a function of temperature. (**A**) Fluorescence emission spectra (λ_{ex} 370 nm) of 200 nM a-NR, 1 µM DnaK, 200 nM DnaJ, 40 nM GrpE, 1 mM ATP and 4 mM ADP at the indicated temperatures. The *dashed line* represents the a-NR spectrum in the absence of the chaperone system at 20°C. (**B**) Spectra of 200 nM a-NR, 1 µM DnaK and 1 mM ADP at the indicated temperatures. The *dashed lines* denote a-NR spectra in the absence of DnaK. (**C**) Plot of $\Delta F/\Delta F'_{max}$ calculated on the basis of λ_{max}^{em} (from spectra as in Figure 3A) versus temperature.

With peptide a-p4 as a substrate, similar data were obtained (Figure 4A-C). Apparently, in the complete DnaK/DnaJ/GrpE chaperone system, a temperature-dependent decrease in the nucleotide exchange activity of GrpE results in a shift of DnaK from its low-affinity T state toward its high-affinity R state.

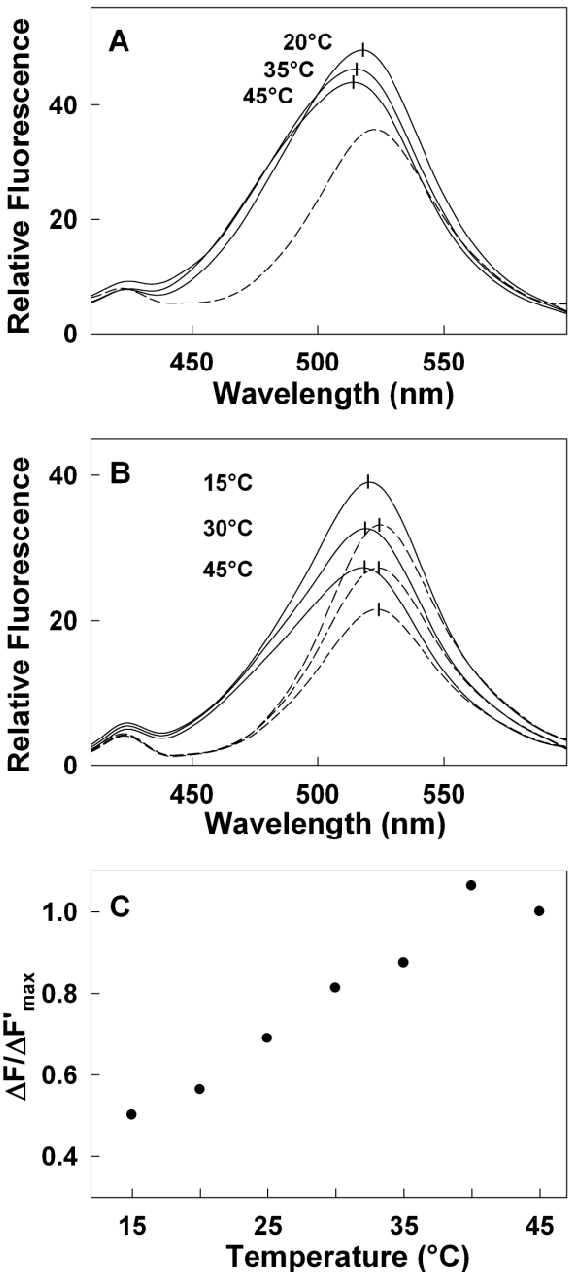

Figure 4 Formation of the DnaK·a-p4 complex in the presence of DnaJ and GrpE as a function of temperature. (**A**) Fluorescence emission spectra (λ_{ex} 370 nm) of 200 nM a-p4, 2 μM DnaK, 200 nM DnaJ, 40 nM GrpE, 1 mM ATP and 4 mM ADP at the indicated temperatures. The *dashed line* represents the a-p4 spectrum in the absence of the chaperone system at 20°C. (**B**) Spectra of 200 nM a-p4, 2 μM DnaK and 1 mM ADP at the indicated temperatures. The *dashed lines* denote a-p4 spectra in the absence of DnaK. (**C**) Plot of $\Delta F/\Delta F'_{max}$ calculated on the basis of λ_{max}^{em} (from spectra as in Figure 4A) versus temperature.

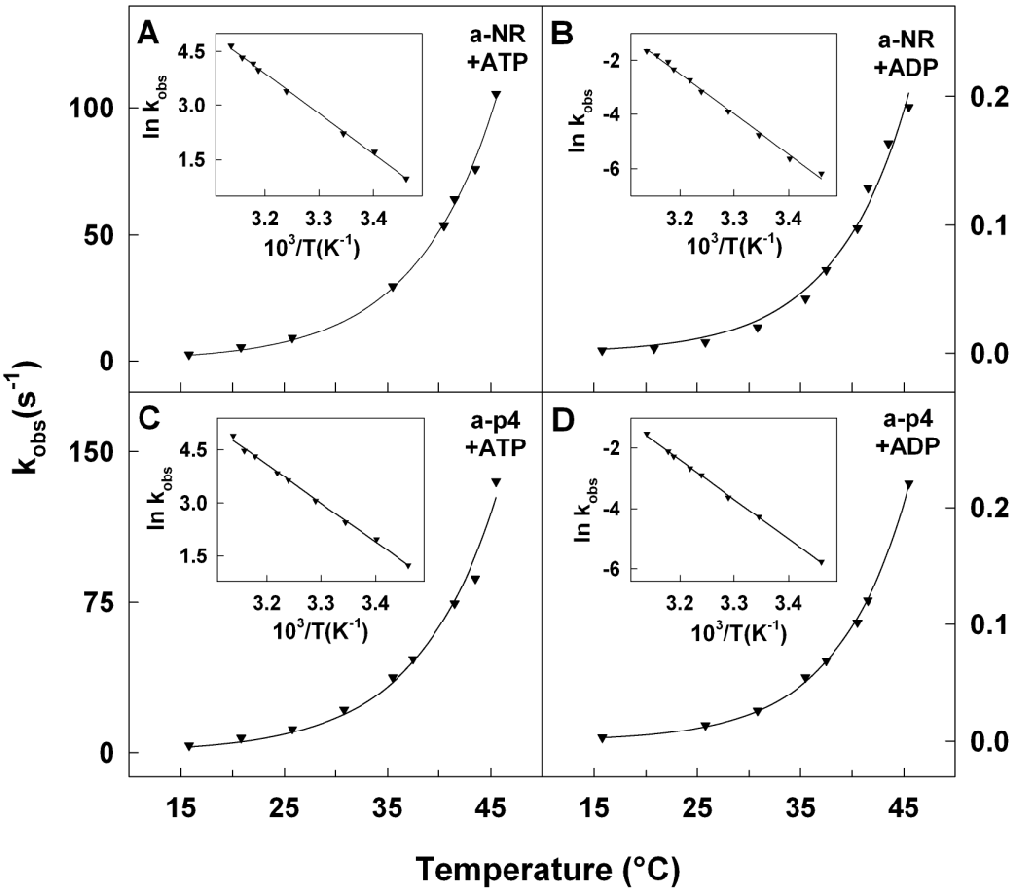

Figure 5 Rate of formation of DnaK·a-peptide complexes as a function of temperature. The following solutions were mixed (final concentrations are indicated): (**A**) [20 µM DnaK, 5 mM ATP] + [500 nM a-NR, 5 mM ATP]. (**B**) [20 µM DnaK, 5 mM ADP] + [500 nM a-NR, 5 mM ADP]. (**C**) [20 µM DnaK, 5 mM ATP] + [500 nM a-p4, 5 mM ATP]. (**D**) [2 µM DnaK, 1 mM ADP] + [200 nM a-p4, 1 mM ADP]. Under all conditions, the reactions were biphasic, the rates of both phases showing Arrhenius temperature dependence. The calculated amplitudes of the first and the second phase with peptide a-NR were 30% and 70% of total, respectively, in the presence of ADP, and 80% and 20% in the presence of ATP; with peptide a-p4 the amplitudes were 15% and 85%, respectively, in the presence of ADP, and 65% and 35% in the presence of ATP. Plots of k_{obs} values of the phase with the higher amplitude versus temperature are shown. The *solid lines* are Arrhenius curves that have been fitted to the k_{obs} values. *Insets* show the Arrhenius plots.

Effect of temperature on the substrate-binding properties of DnaK in the absence of the co-chaperones – In the above experiments with the complete chaperone system (Figure 3 and 4) we determined the fraction of R-state DnaK by the extent of formation of DnaK·a-peptide complexes. To confirm that the increased sequestering of peptide at higher temperature is due to the co-chaperones modulating the ATPase cycle rather than due to a change in the intrinsic substrate-binding properties of DnaK, we investigated the formation of the DnaK·a-peptide complexes in the absence of the co-chaperones at varying temperatures.

Table I Dissociation equilibrium constants of the DnaK·a-NR complex at different temperatures. The values were determined by fluorescence titration of 50 nM a-NR in assay buffer containing 1 mM ATP or ADP with up to 35 μM or up to 2.5 μM DnaK, respectively. Averaged K_d values from 2 or 3 measurements are shown.

Temperature (°C)	K_d (μM)	
	With ATP	With ADP
25	36	0.38
35	17	0.38
45	14	0.56

We found the rates of complex formation with peptides a-NR or a-p4 to follow an Arrhenius temperature dependence in the presence of ATP or ADP (Figure 5), indicating that the mechanism of complex formation remains the same from 15°C to 45°C. These findings are consistent with previously reported rate constants of DnaK for fluorescence-labeled Cro peptide between 5°C and 37°C (Farr et al., 1995). The dissociation equilibrium constants of the DnaK·a-NR complex in the presence of ATP or ADP did not significantly change between 25°C and 45°C (Table I). Thus, the observed shift of DnaK toward its R state can not be explained by the temperature-dependence of the substrate binding properties of DnaK itself.

Reversibility of the shift of DnaK toward its high-affinity R state – To examine whether the temperature-induced shift of DnaK toward its high-affinity R state is indeed controlled by the thermosensor action of GrpE (Grimshaw et al., 2001, 2003; Groemping and Reinstein, 2001; Gelinas et al., 2002, 2003), we omitted GrpE in the experiment. In the absence of GrpE, the spectra at 15°C and 45°C showed no difference in λ_{max}^{em} (Figure 6A), indicating that the observed temperature-dependent shift of DnaK toward its R state (Figure 3A and 4A) depends on the presence of GrpE. The shift toward the R state proved to be fully reversible upon lowering the temperature (Figure 6B), consistent with the reversibility of the thermal transition of GrpE reported previously (Grimshaw et al., 2001).

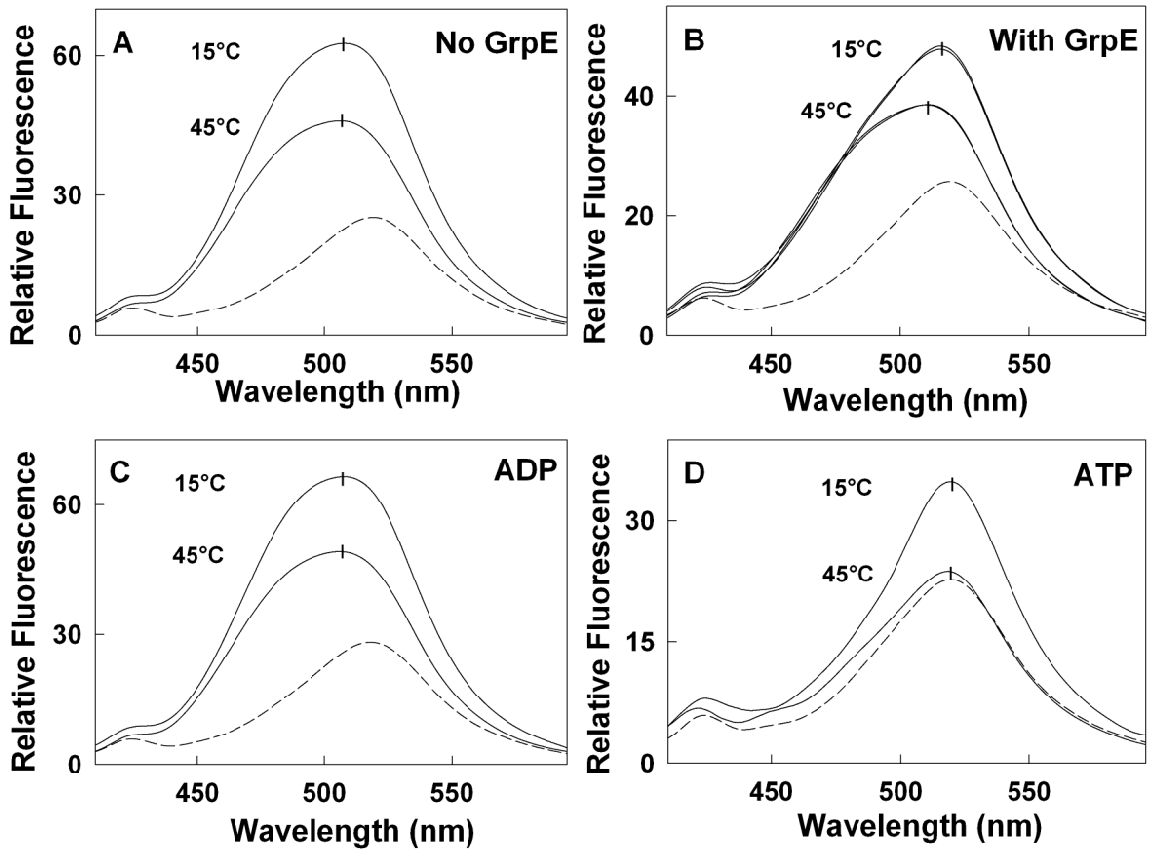

Figure 6 Formation of the DnaK·a-NR complex in the presence and absence of GrpE as a function of temperature. (**A**) Fluorescence emission spectra (λ_{ex} 370 nm) of 200 nM a-NR, 1 µM DnaK, 200 nM DnaJ, 1 mM ATP and 4 mM ADP at 15°C and 45°C. The *dashed line* denotes the a-NR spectrum in the absence of chaperones at 45°C. (**B**) Same as A, but in the presence of 40 nM GrpE. The same solution was heated from 15°C to 45°C, cooled to 15°C and reheated to 45°C. (**C**) Spectra of 200 nM a-NR, 1 µM DnaK, 200 nM DnaJ, 40 nM GrpE and 4 mM ADP at 15°C and 45°C. The *dashed line* denotes the a-NR spectrum in the absence of chaperones at 45°C. (**D**) Same as C, but in the presence of 1 mM ATP instead of ADP.

The first and second spectrum at 15°C as well as the two spectra at 45°C were superimposable; the effect of ATP hydrolysis during the experiment appears to be negligible. The functional adaptation of the DnaK system, both to high and low temperature, was found to be already completed within the time period used for attaining the set temperature.

If either ATP or ADP alone was present, no shift of λ_{max}^{em} was observed and the decrease in fluorescence intensity upon increasing temperature was similar to that found in the absence of the co-chaperones (Figure 6C and D). In the presence of ADP, only R-state DnaK can be formed. In the presence of ATP, DnaK persists predominantly in the T state because

under the chosen conditions the GrpE-catalyzed ADP/ATP exchange is both at low and high temperature much faster than the DnaJ-stimulated hydrolysis of ATP, i.e. the T→R conversion.

Concluding remarks

The present study shows that temperature directly modulates the functional properties of the DnaK/DnaJ/GrpE chaperone system. A mere increase in temperature suffices to shift DnaK toward its high-affinity R state. This thermal response is due to the divergent temperature-dependence of the two co-chaperones. While DnaJ, which stimulates the hydrolysis of DnaK-bound ATP (see Figure 1), follows an Arrhenius temperature dependence, the ADP/ATP exchange activity of the thermosensor GrpE increases less and less with increasing temperature and even decreases above 40°C (Grimshaw et al., 2001). The higher fraction of R-state DnaK increases the fraction of substrate being sequestered by DnaK. We postulate this immediate heat shock response to be relevant to the cell where the ATPase cycle of DnaK also exists in a co-chaperone-controlled steady state (Mayer et al., 2001). During heat shock, an increase in the fraction of DnaK-bound target protein may be expected to reduce protein aggregation. Mutational analysis in the ATPase domain have indicated that even small disturbances in the ADP/ATP exchange rate suffice to affect the chaperone activity of DnaK (Brehmer et al., 2001).

In *E. coli*, the expression level of the DnaK chaperone system is regulated by the alternative sigma factor 32 (σ^{32}), which directs RNA polymerase to transcribe this particular set of genes. Within 5 min after a temperature upshift, the rate of synthesis of the heat shock proteins increases 10 to 20-fold resulting in an approximately 2-fold increase in the cellular concentrations of DnaK, DnaJ and GrpE (Connolly et al., 1999). The functional adaptation of the extant chaperone system, as reported here, precedes the enhanced expression of its components. The immediate heat shock response might serve to bridge the time lag of the

σ^{32}-mediated heat shock response. The instant reversibility upon normalization of temperature might be another important feature of this rapidly deployable protection mechanism. Similar to *E. coli* GrpE, its homolog in the thermophilic bacterium *Thermus thermophilus* has been found to undergo a reversible thermal transition within the physiological temperature range of that organism (Groemping and Reinstein, 2001). The occurrence of a thermosensor action of GrpE in both an archaeon and an eubacterium emphasizes the importance of the immediate heat shock response in prokaryotes.

This study provides unequivocal evidence for the existence of a GrpE-mediated T→R shift of DnaK at heat-shock temperatures. Future experiments will have to establish the significance of this mechanistic feature for the chaperone effect on proteins.

Acknowledgments

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The importance of having thermosensor control in the DnaK chaperone system

Rahel K. Siegenthaler and Philipp Christen

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Abstract

In addition to the σ^{32}-mediated heat shock response, the DnaK/DnaJ/GrpE molecular chaperone system of *Escherichia coli* directly adapts to elevated temperatures by sequestering a higher fraction of substrate. This immediate heat shock response is due to the differential temperature dependence of the activity of DnaJ, which stimulates the hydrolysis of DnaK-bound ATP, and the activity of GrpE, which facilitates ADP/ATP exchange and converts DnaK from its high-affinity ADP-liganded state into its low-affinity ATP-liganded state. GrpE acts as thermosensor, its ADP/ATP exchange activity decreasing above 40°C. To assess the importance of this reversible thermal adaptation for the chaperone action of the DnaK/DnaJ/GrpE system during heat shock, we used glucose-6-phosphate dehydrogenase and luciferase as substrates. We compared the performance of wild-type GrpE as component of the chaperone system with that of GrpE(R40C). In this mutant, the thermosensing helices are stabilized with an intersubunit disulfide bond and its nucleotide exchange activity thus increases continuously with increasing temperature. Wild-type GrpE with intact thermosensor proved superior to GrpE(R40C) with desensitized thermosensor. The chaperone system with wild-type GrpE yielded not only a higher fraction of refolding-competent protein at the end of a heat shock, but also protected luciferase more efficiently against inactivation during heat shock. Consistent with their differential thermal behavior, the protective effects of wild-type GrpE and GrpE(R40C) diverged more and more with increasing temperature. Thus, the direct thermal adaptation of the DnaK chaperone system by thermosensing GrpE is essential for efficient chaperone action during heat shock.

Introduction

Molecular chaperones of the 70-kDa heat shock protein (Hsp70) family participate in many cellular processes, including the folding, membrane translocation and degradation of proteins

(Hartl and Hayer-Hartl, 2002). The chaperones recognize and interact with hydrophobic peptide segments, which are exposed by nascent polypeptide chains during synthesis and by misfolded proteins during stress, in particular heat shock. The irreversible formation of protein aggregates is thus reduced, increasing the yield of properly folded and refolded native protein. Hsp70 bind and release their substrates in an ATP-driven cycle (McCarty et al., 1995; Theyssen et al., 1996). The ATPase activity resides in the NH_2-terminal domain (Flaherty et al., 1990; Harrison et al., 1997) and modulates the substrate binding properties of the COOH-terminal peptide-binding domain (Zhu et al., 1996). The ATP-liganded T state is characterized by low affinity for substrates and fast rates of binding and release, whereas the ADP-liganded R state shows high affinity for substrates with slow kinetics (Palleros et al., 1993; Schmid et al., 1994). DnaK, an Hsp70 homolog in *E. coli*, acts in concert with its co-chaperones DnaJ, an Hsp40 homolog, and GrpE (Liberek et al., 1991; McCarty et al., 1995; Pierpaoli et al., 1998). DnaJ stimulates the hydrolysis of DnaK-bound ATP and converts T-state DnaK into its high-affinity R state (Figure 1). GrpE facilitates ADP/ATP exchange and reconverts DnaK into the low-affinity T state. In the presence of ATP, the conjoint action of the two co-chaperones DnaJ and GrpE controls the steady-state distribution of T-state and R-state DnaK and thus modulates the fraction of substrate sequestered by high-affinity R-state DnaK (Liberek et al., 1991; Pierpaoli et al., 1998; Siegenthaler et al., 2004).

In a study on the temperature dependence of the structural and functional properties of the individual components of the DnaK chaperone system (Grimshaw et al., 2001), GrpE has been found to undergo a reversible, thermal transition in the physiologically relevant temperature range (transition midpoint at ~48°C), as observed with circular dichroism measurements and differential scanning calorimetry. In DnaK and DnaJ, no conformational changes have been observed between 15°C and 48°C.

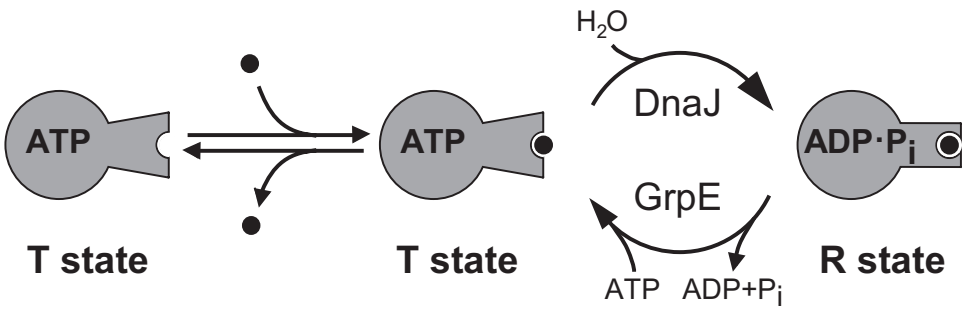

Figure 1 The DnaK/DnaJ/GrpE chaperone cycle. The substrate is fed into the cycle by fast binding to ATP-liganded T-state DnaK. Direct binding to ADP-liganded R-state DnaK is too slow to be of physiological significance (Schmid et al., 1994; McCarty et al., 1995). DnaJ-stimulated hydrolysis of DnaK-bound ATP locks DnaK onto the substrate (Laufen et al., 1999; Han and Christen, 2003); direct release of substrate from ADP-liganded R-state DnaK is negligibly slow (Schmid et al., 1994). GrpE-catalyzed ADP/ATP exchange completes the cycle and releases the substrate. Under heat shock conditions, the substrate is dynamically sequestered by high-affinity DnaK due to a shift of the co-chaperone-controlled steady state of the system towards the R state (Grimshaw et al., 2003; Siegenthaler et al., 2004).

Consistent with the structural data, the rate of the DnaJ-triggered T→R conversion follows an Arrhenius temperature dependence, while the rate of the GrpE-dependent R→T conversion increases less and less with increasing temperature and even decreases above 40°C (Grimshaw et al., 2001). Similar studies of GrpE and its homolog from *Thermus thermophilus* have confirmed the occurrence of a reversible thermal transition (Groemping and Reinstein, 2001; Gelinas et al., 2002). Stabilization of the pair of the long NH_2-terminal helices in the GrpE dimer with an engineered disulfide bond (R40C; Figure 2) abolishes the thermal transition in GrpE and reduces the deviation of the ADP/ATP exchange activity from an Arrhenius temperature dependence, indicating that the long helix pair acts as the primary thermosensor of the chaperone system (Grimshaw et al., 2003; Gelinas et al., 2003).

In *E. coli*, DnaK and its co-chaperones are constitutively expressed as well as induced by heat shock or other cellular stress. This familiar heat shock response is mediated by sigma factor 32 (σ^{32}), which directs RNA polymerase to transcribe the particular set of heat shock genes. Within minutes after heat shock, the cellular concentrations of DnaK, DnaJ and GrpE are increased approximately 2-fold (Connolly et al., 1999; Liu, 2002).

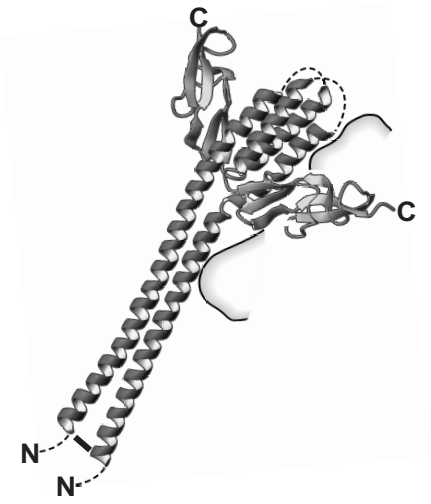

Figure 2 The nucleotide exchange factor GrpE. The GrpE(G122D) dimer has been co-crystallized with the ATPase domain of DnaK (Harrison et al., 1997). To date the crystal structure of wt GrpE itself is not available. α-Helices and β-sheets in GrpE are represented by *ribbons*, and residues not resolved in the crystal structure are denoted with *dashed lines*. The ATPase domain of DnaK is indicated with a *shaded contour*. The *solid bar* indicates the position of the newly introduced disulfide bond in GrpE(R40C). *C*, COOH-terminus; *N*, NH_2-terminus. The atomic coordinates are from Protein data bank entry 1DKG.

Recent experiments with the isolated DnaK/DnaJ/GrpE/ATP chaperone system have shown that a mere increase in temperature suffices to sequester a higher fraction of fluorescence-labeled peptide substrates (Siegenthaler et al., 2004). This direct thermal response of the chaperone system is due to the differential temperature dependence of the two co-chaperones, resulting in a shift toward R-state DnaK with high affinity for substrates. Here, we compare the co-chaperone efficiencies of wild-type (wt) GrpE with intact thermosensor and GrpE(R40C) with desensitized thermosensor during heat shock. The DnaK system with wt GrpE proved superior to that with GrpE(R40C) in protecting protein substrate against both heat inactivation and aggregation, indicating the importance of the GrpE thermosensor for efficient chaperone action under heat shock conditions.

Experimental procedures

Materials – DnaK was expressed and purified as described previously (Feifel et al., 1996). Its concentration was determined photometrically with a molar absorption coefficient of

ε_{280} = 14,500 $M^{-1}cm^{-1}$ (Hellebust et al., 1990). Purified DnaK contained less than 0.1 mol nucleotide/mol DnaK (Feifel et al., 1996) and was stored in assay buffer (25 mM Hepes/NaOH, 100 mM KCl, 10 mM $MgCl_2$, pH 7.4) at –80°C. DnaJ and wt GrpE, prepared as reported elsewhere (Schönfeld et al., 1995a; Schönfeld et al., 1995b), were a gift from Dr. H.-J. Schönfeld, Basel. The stock solutions in 50 mM Tris/HCl, 100 mM NaCl, pH 7.7 were kept at –80°C. The concentrations of DnaJ and wt GrpE were determined with ε_{277} = 18,100 $M^{-1}cm^{-1}$ and ε_{279} = 2,720 $M^{-1}cm^{-1}$, respectively. Mutant GrpE(R40C) was expressed and purified as described previously (Grimshaw et al., 2003); its concentration was determined by amino acid analysis. Throughout this report the indicated protein concentrations refer to the protomer. Apyrase (grade III) from potato was purchased from Sigma; its stock solution in water (1 unit/µl) was stored at –80°C. ATP-Na_2 (purity >98%) and *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (G6PDH) were from Fluka; *Photinus pyralis* luciferase was from Roche. The concentration of G6PDH, stored in 6 M guanidine hydrochloride, 50 mM Tris/HCl, pH 7.6, at –20°C, was determined by the Bradford method with bovine serum albumin as standard. Luciferase (1 mg/ml) was stored in 0.5 M Tris/acetate pH 7.5 at –80°C as recommended by the manufacturer.

Prevention of protein aggregation during heat shock measured by after-heat-shock refolding in the presence of the complete chaperone system − Thermostable G6PDH, a dimeric enzyme of 104 kDa, was denatured in 6 M guanidine hydrochloride, 50 mM Tris/HCl, pH 7.6. For heat treatment, the G6PDH solution was prediluted into assay buffer and added (40 µl) to test tubes on ice containing various combinations of the components of the DnaK/DnaJ/GrpE/ATP system in 30 µl, and incubated at 45°C. Guanidine hydrochloride was thereby 94 times diluted. After heat shock, the lacking components of the chaperone system were added (30 µl) and the solution incubated at 25°C. The experiments with

luciferase, a thermolabile, monomeric enzyme of 60 kDa, were performed the same way except that luciferase was directly denatured by heat. The temperature was controlled with a water bath (± 0.1°C). The extent of ATP hydrolysis during the time course of the experiments was determined as described (Pierpaoli et al., 1998) and found to be negligible (not shown).

Chaperone-assisted refolding of heat-treated protein – G6PDH, denatured in guanidine hydrochloride, was prediluted into assay buffer containing DnaK and DnaJ (for details, see legend to Figure 4C), and incubated at 48°C. After heat shock, the solution (70 µl) was added to test tubes on ice containing ATP and GrpE (wt or R40C) at varying concentrations in 30 µl, and incubated at 25°C. The experiments with wt GrpE and GrpE(R40C) were performed in parallel. The refolding experiments with luciferase were performed likewise.

Prevention of protein aggregation during heat shock measured by spontaneous after-heat-shock refolding – G6PDH, denatured in guanidine hydrochloride, was prediluted into assay buffer containing DnaK and DnaJ. The solution (70 µl) was then added to test tubes on ice containing ATP and GrpE (wt or R40C) at varying concentrations in 30 µl, and incubated at heat shock temperature. After heat treatment, 5 units of apyrase (5 µl) were added to deplete the chaperone system of ATP, and the solution incubated at 25°C. The experiments with luciferase were performed the same way.

Retardation of luciferase inactivation during heat shock – Luciferase was prediluted into assay buffer containing DnaK and DnaJ, added (70 µl) to test tubes on ice containing ATP and GrpE at varying concentrations in 30 µl, and incubated at heat shock temperature.

Enzyme activity measurements – The activity of G6PDH was determined photometrically by measuring the increase in NADH concentration at 340 nm during 30 s with an HP 8453 spectrophotometer at 25°C. The assay volume was 1 ml and contained 3 mM glucose-6-phosphate and 0.15 μM NAD^+ in 200 mM Hepes/NaOH, pH 7.6. The reaction was started by adding 15 μl of the G6PDH-containing sample solution. Luciferase activity was measured in a Lumac Biocounter M1500 as described previously (Bischofberger et al., 2003).

Results and discussion

The DnaK chaperone system prevents protein aggregation during heat shock – Upon heat shock, labile proteins are denatured and either are irreversibly trapped in aggregates or remain in a refolding-competent state. The formation of protein aggregates upon heat denaturation has been measured *in vitro* by several methods, including sedimentation analysis, light scattering and Congo red or thioflavin T staining (Schröder et al., 1993; Goloubinoff et al., 1999; Ben-Zvi et al., 2004; Watanabe and Yoshida, 2004). The fraction of aggregated protein that is detected depends on the analytical method used as well as the solubility, size and shape of the aggregates formed. In this study, we used enzymes as substrates and determined the fraction of refolding-competent protein rather than that of aggregated protein, i.e. we measured the amount of active enzyme recovered after heat shock. To assess the effect of the individual components of the DnaK chaperone system on preventing protein aggregation during heat shock, we subjected the protein substrate to heat shock in the presence of different combinations of chaperones and then let it refold with the complete DnaK/DnaJ/GrpE/ATP chaperone system present. Any observed differences in the yield of refolding could thus be unequivocally attributed to the differential conditions during heat shock. As protein substrates we used G6PDH and luciferase.

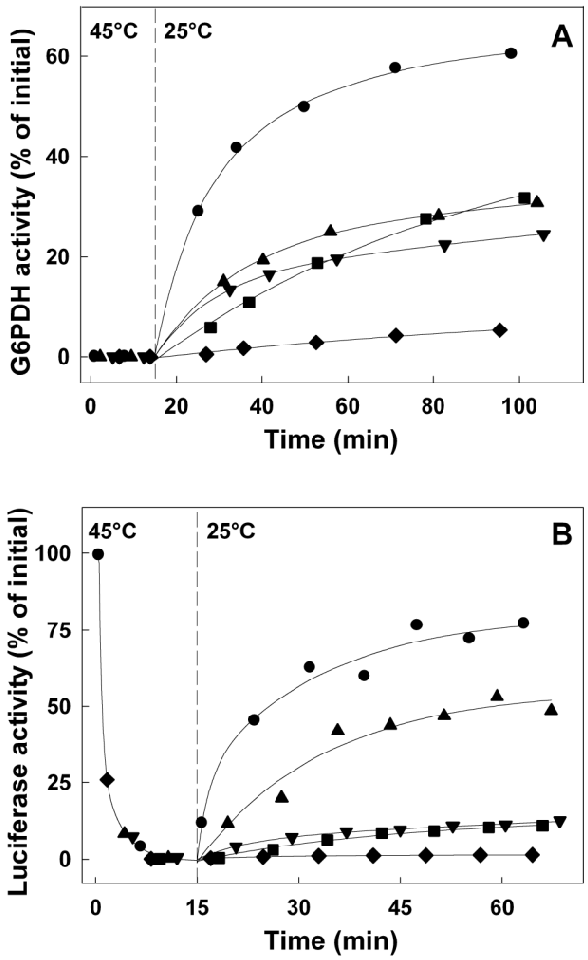

Figure 3 The DnaK chaperone system prevents protein aggregation during heat shock. (**A**) Chemically denatured G6PDH (see Experimental procedures) was incubated at 45°C for 15 min in the presence of the indicated components of the chaperone system: DnaK, DnaJ, GrpE and ATP (●), DnaK, DnaJ and GrpE (■), DnaK, DnaJ and ATP (▲), DnaK, GrpE and ATP (▼), and no chaperone, no ATP (◆). Final concentrations (as indicated throughout) were 250 nM G6PDH, 3.5 µM DnaK, 0.7 µM DnaJ, 0.35 µM GrpE and 10 mM ATP. After heat shock, the samples were complemented with the lacking components of the DnaK/DnaJ/GrpE/ATP chaperone system and incubated at 25°C. The enzymic activity of G6PDH was measured during and after heat shock at different time points and expressed as percentage of the initial activity. The data points were fitted with a double-exponential equation. (**B**) An analogous experiment was performed with 250 nM luciferase. In the case of heat-labile luciferase, the chemical denaturation preceding the heat shock was omitted.

Both enzymes had previously been shown to form stable protein aggregates at heat shock temperatures, as observed with size exclusion chromatography and light scattering (Schröder et al., 1993; Diamant et al., 2000). G6PDH, which is heat-stable, was denatured in 6 M guanidine hydrochloride. After dilution of the denaturant, G6PDH was exposed to a temperature of 45°C for 15 min in the presence of various combinations of the components of the chaperone system. During the heat shock, G6PDH remained inactive (Figure 3A; see also

Diamant and Goloubinoff, 1998). After heat shock, G6PDH was reactivated at 25°C in the presence of the fully active chaperone system to ~60% of the activity prior to denaturation if the complete chaperone system including ATP had already been present during heat shock. If ATP had been absent during heat shock, the yield of active enzyme was only ~30% of the initial. The same yield was obtained in the absence of either DnaJ or GrpE during heat shock. When no chaperones had been present during heat shock, the fraction of refolding-competent G6PDH was less than 10%.

In analogous experiments, thermolabile luciferase (Schröder et al., 1993) was directly inactivated by heat denaturation at 45°C (Figure 3B). After heat shock, luciferase was reactivated at 25°C to ~75% of the initial activity, if the complete chaperone system including ATP had been present during heat shock. Almost no refolding occurred if DnaJ or ATP had been absent during heat shock. In the absence of GrpE during heat shock, the fraction of refolding-competent luciferase decreased to ~50%. Apparently, the prevention of aggregation of both G6PDH and luciferase is a dynamic process requiring a continuously running T-R cycle of DnaK. The control of the ATP-driven chaperone cycle not only by DnaJ (Laufen et al., 1999; Han and Christen, 2003) but also by GrpE (Liberek et al., 1991; Pierpaoli et al., 1998) is essential for maximum protection of substrate protein against aggregation.

Thermosensor effect of GrpE in the prevention of protein aggregation during heat shock – Under heat shock conditions, the differential temperature dependence of the activities of the co-chaperones DnaJ and GrpE becomes manifest. The rate of the DnaJ-stimulated T→R conversion increases exponentially with increasing temperature whereas the rate of the GrpE-facilitated R→T conversion increases less and less and even decreases above 40°C (Grimshaw et al., 2001). The resulting shift of DnaK toward its R state increases the fraction of substrate that is sequestered by high-affinity DnaK (Siegenthaler et al., 2004).

In a first series of experiments, we investigated the protective effect of the chaperone system against protein aggregation as a function of GrpE concentration. To focus on the heat shock phase and to minimize the chaperone effect on the protein substrates during the refolding phase, we inhibited the chaperone system after the heat shock by depleting it of ATP and measured the amount of spontaneously refolding protein substrate (instead of providing identical refolding conditions after heat shock as in Figure 3). For removing ATP, we used apyrase, an ATP diphosphohydrolase that hydrolyzes ATP into AMP and inorganic phosphate. Apyrase completely inhibits the chaperone-assisted refolding (Figure 4F). Chemically denatured G6PDH was subjected to a temperature of 48°C for 15 min in the presence of DnaK, DnaJ, ATP and varying GrpE concentrations. Subsequently, apyrase was added and the spontaneous refolding of G6PDH at 25°C monitored. The fraction of refolding-competent G6PDH depended on the concentration of GrpE (Figure 4A). The maximum yield of reactivated G6PDH was obtained at a GrpE concentration of ~1 μM (Figure 4B); both lower and higher GrpE concentrations resulted in a lower amount of refolded G6PDH. GrpE shifts the steady state of the DnaK ATPase cycle from the R state toward the T state. Apparently, maximum protection of G6PDH against aggregation requires an optimum steady-state R state/T state ratio. The thermosensor control of GrpE may thus be assumed to have an effect on the prevention of protein aggregation during heat shock, provided that a temperature-induced decrease in nucleotide exchange activity of GrpE (see Figure 4E) has the same effect as a decrease in its concentration.

In the following series of experiments, we examined with a similar set-up the role of the thermosensor action of GrpE in the protection of protein substrate against aggregation during heat shock. We compared the co-chaperone efficiency of wt GrpE with that of GrpE(R40C) (Figure 2), in which the pair of the thermosensing helices had been stabilized with an intersubunit disulfide bond at their NH_2-termini (Grimshaw et al., 2003). In GrpE(R40C), no thermal transition is observed in the physiological temperature range.

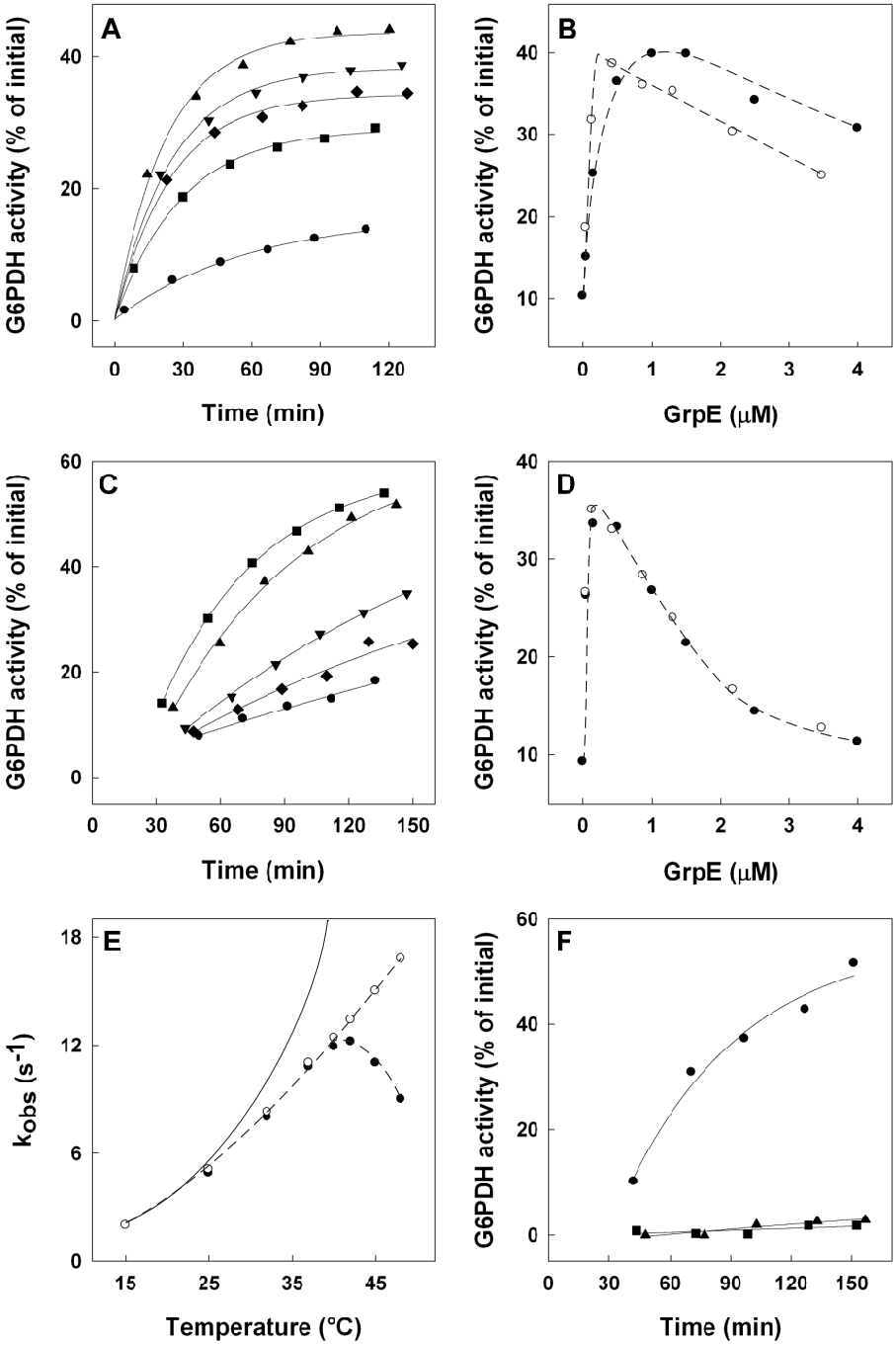

Figure 4 Thermosensor effect of GrpE in the prevention of G6PDH aggregation during heat shock. (**A**) Chemically denatured G6PDH (250 nM) was incubated at 48°C for 15 min in the presence of 3.5 μM DnaK, 0.7 μM DnaJ, 10 mM ATP plus wt GrpE or GrpE(R40C) at the indicated concentrations: 0 (●), 0.15 (■), 1.0 (▲), 2.5 (▼), and 4.0 μM wt GrpE (◆); the data with GrpE(R40C) are not shown. After heat shock, 5 units of apyrase were added and the samples incubated at 25°C. Enzymic activity of spontaneously refolding G6PDH was measured at different time points. The data points were fitted with a single-exponential equation (not all experiments are shown for clarity's sake). (**B**) The interpolated activity at 60 min (obtained from data as shown in Panel A) was plotted as a function of the concentration of wt GrpE (●) and GrpE(R40C) (○). The actual concentrations of GrpE(R40C) were 1.2 times higher than the indicated values (for explanation, see legend to Figure 4D). (**C**) G6PDH (250 nM) was incubated at 48°C for 15 min in the presence of 3.5 μM DnaK and 0.7 μM DnaJ (without DnaK and DnaJ, only a minor fraction of G6PDH refolded after heat shock, see Figure 3).

The temperature dependence of the ADP/ATP exchange activity of wt and disulfide-stabilized GrpE is similar between 15°C and 40°C; above this temperature range, the stabilizing effect of the interhelical crosslink in GrpE(R40C) becomes manifest and the rate of the R→T conversion increases continuously (Grimshaw et al., 2003), resulting in an increasing difference in nucleotide exchange activity between wt and mutant GrpE with increasing temperature (Figure 4E). To normalize their co-chaperone activities at low temperature, we monitored the refolding of heat-treated G6PDH at 25°C as a function of the concentration of wt GrpE and GrpE(R40C) in the presence of DnaK, DnaJ and ATP. The yield of enzymic activity was found to be GrpE-dependent (Figure 4C), consistent with previous studies (Packschies et al., 1997; Diamant and Goloubinoff, 1998; Ben-Zvi et al., 2004). If the concentration of GrpE(R40C) was divided by 1.2, the reactivation pattern of G6PDH in the presence of GrpE(R40C) coincided with that in the presence of wt GrpE (Figure 4D). Apparently, GrpE(R40C) possesses at 25°C a 1.2 times lower co-chaperone activity than wt GrpE, consistent with its previously observed somewhat lower nucleotide exchange activity (Grimshaw et al., 2003). To examine whether desensitization of the

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After heat shock, GrpE (wt or R40C) at the indicated concentrations and 10 mM ATP (that both had separately been heat-shocked at 48°C for 15 min) were added and the samples incubated at 25°C: 0 (●), 0.15 (■), 1.0 (▲), 2.5 (▼), and 4.0 μM wt GrpE (◆); data with GrpE(R40C) are not shown. The activity of refolding G6PDH was measured at different time points. The initial lag in refolding (see also Diamant and Goloubinoff, 1998; Ben-Zvi et al., 2004) was not included in the fitting. (**D**) The enzymic activity of G6PDH at 60 min (obtained from data as shown in Panel C) was plotted as a function of the concentration of wt GrpE (●) and GrpE(R40C) (○). The reactivation pattern in the presence of GrpE(R40C) was brought to coincidence with that in the presence of wt GrpE by dividing the actual GrpE(R40C) concentrations by 1.2 (for detailed explanation, see text). (**E**) Co-chaperone activity of wt GrpE (●) and GrpE(R40C) (○) as a function of temperature. The rates of the GrpE-dependent R→T conversion were determined by monitoring the decrease in intrinsic fluorescence of DnaK at the indicated temperatures with a stopped-flow device (data taken from Grimshaw et al., 2003). The indicated k_{obs} values are those of wt GrpE. The values of GrpE(R40C) were normalized based on the value of wt GrpE at 15°C. For comparison, the *solid line* corresponding to Arrhenius temperature dependence was calculated on the basis of the rates at 15°C and 25°C. (**F**) Control showing the efficacy of apyrase in inhibiting the chaperone system. G6PDH (250 nM), 3.5 μM DnaK and 0.7 μM DnaJ were incubated at 48°C for 15 min, then 0.35 μM GrpE plus 10 mM ATP or buffer were added, and the samples incubated at 25°C. Subsequently, either 5 units of apyrase or buffer were added and the enzymic activity of spontaneously refolding G6PDH was measured at different time points. GrpE, ATP + buffer (●), GrpE, ATP + apyrase (■), and buffer + buffer (▲).

thermosensor in GrpE affected the prevention of protein aggregation during heat shock, we performed the experiment of Figure 4B with GrpE(R40C) instead of wt GrpE. At GrpE concentrations >1 μM, the yield of refolding-competent G6PDH was lower in the presence of GrpE(R40C) than in the presence of wt GrpE, the wt co-chaperone with intact thermosensor being superior to the desensitized co-chaperone in the prevention of protein aggregation (Figure 4B). The GrpE(R40C) concentration for maximum protective effect was slightly shifted to lower concentration, because the ADP/ATP exchange activity of GrpE(R40C) at heat shock temperature is higher than that of wt GrpE (Figure 4E). Accordingly, at GrpE concentrations <1 μM, the yield of reactivated G6PDH was higher in the presence of GrpE(R40C) than in the presence of wt GrpE.

The intracellular concentrations of the chaperones *in vivo* are considerably higher than the concentrations used in this study. DnaK, DnaJ and GrpE have been reported to represent 1.4%, 0.02% and 0.14% of total cellular protein, respectively (Neidhardt and VanBogelen, 1987). The concentration of DnaK has been estimated to be in the range of 100 μM (Hartl et al., 1992). Accordingly, the concentrations of DnaJ and GrpE may be estimated to be 1.4 μM and 10 μM, respectively. The 7-fold molar excess of GrpE over DnaJ is in the same range as the intracellular GrpE/DnaJ molar ratio of 2.5 determined recently in our laboratory (Liu, 2002). In the present study with the DnaJ concentration invariably set at 0.7 μM, this GrpE/DnaJ molar ratio corresponds to the conditions at GrpE concentrations >1 μM, where wt GrpE was found to be superior to GrpE(R40C) (Figure 4B).

When luciferase was used as substrate instead of G6PDH, similar results were obtained (Figure 5A-D). Again, the thermosensor control of GrpE that reduces its ADP/ATP exchange activity at higher temperatures, proved beneficial for preventing protein aggregation during heat shock. The effect of thermosensor control may be assumed to exceed the effect evident in these experiments (see comments under Concluding remarks).

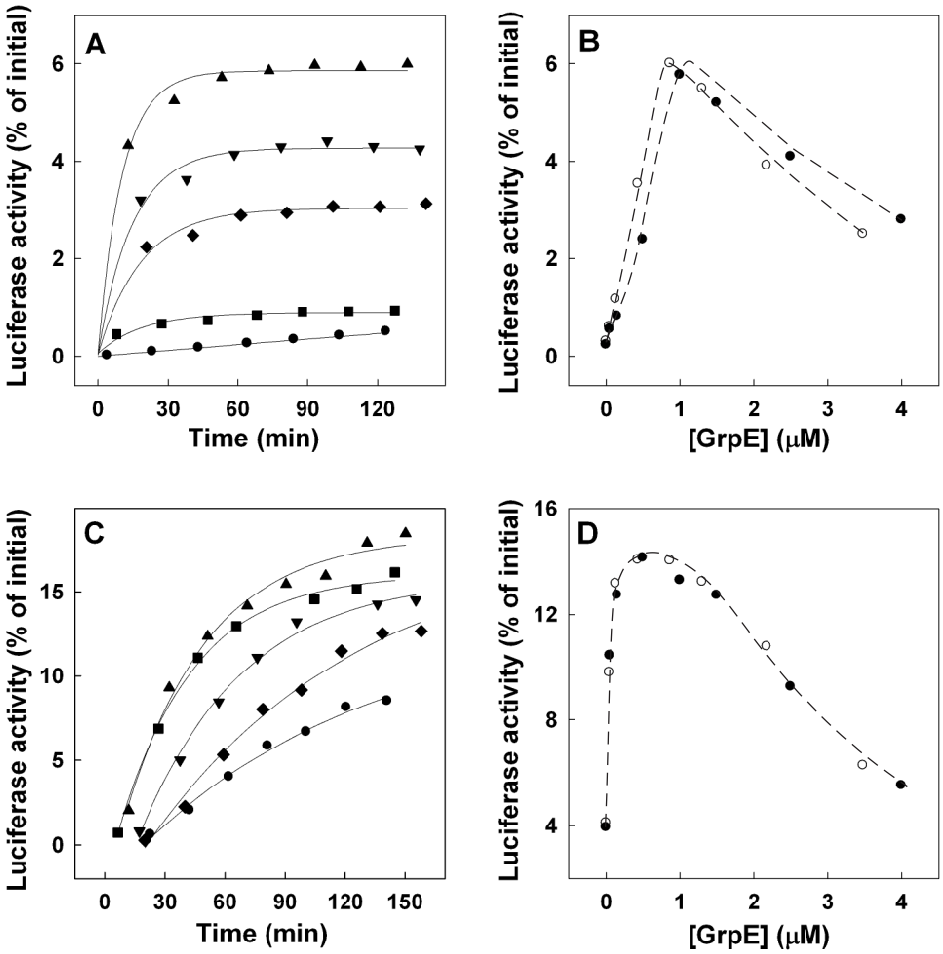

Figure 5 Thermosensor effect of GrpE in the prevention of luciferase aggregation during heat shock. Experiments analogous to those in Figure 4 were performed with luciferase as substrate. (**A**) Luciferase (250 nM), 3.5 μM DnaK, 0.7 μM DnaJ, 10 mM ATP plus wt GrpE or GrpE(R40C) at the indicated concentrations were incubated at 48°C for 15 min, 5 units of apyrase were added and the samples incubated at 25°C: 0 (●), 0.15 (■), 1.0 (▲), 2.5 (▼), and 4.0 μM wt GrpE (◆); the data with GrpE(R40C) are not shown. Control experiments of ATP depletion by apyrase are not shown. (**B**) Luciferase activity at 60 min (obtained from data as shown in Panel A) was plotted as a function of the concentration of wt GrpE (●) and GrpE(R40C) (○). The concentrations of GrpE(R40C) were 1.2 times higher than indicated (for explanation, see legend to Figure 5D). (**C**) Luciferase (250 nM), 3.5 μM DnaK and 0.7 μM DnaJ were exposed to 48°C for 15 min, then added to samples containing 10 mM ATP plus wt GrpE or GrpE(R40C) at the indicated concentrations, and incubated at 25°C: 0 (●), 0.15 (■), 1.0 (▲), 2.5 (▼), and 4.0 μM wt GrpE (◆); the data with GrpE(R40C) are not shown. (**D**) Plot of luciferase activity at 60 min (obtained from data as shown in Panel C) *versus* the concentration of wt GrpE (●) and GrpE(R40C) (○). The reactivation pattern in the presence of GrpE(R40C) was brought to coincidence with that in the presence of wt GrpE by dividing the actual GrpE(R40C) concentrations by 1.2 (for detailed explanation, see text).

Efficiency of the thermosensor control by GrpE as a function of temperature – The rate of the wt GrpE-catalyzed R→T conversion of DnaK progressively decreases above 40°C with increasing temperature, whereas in the presence of GrpE(R40C) the rate continues to increase (see Figure 4E).

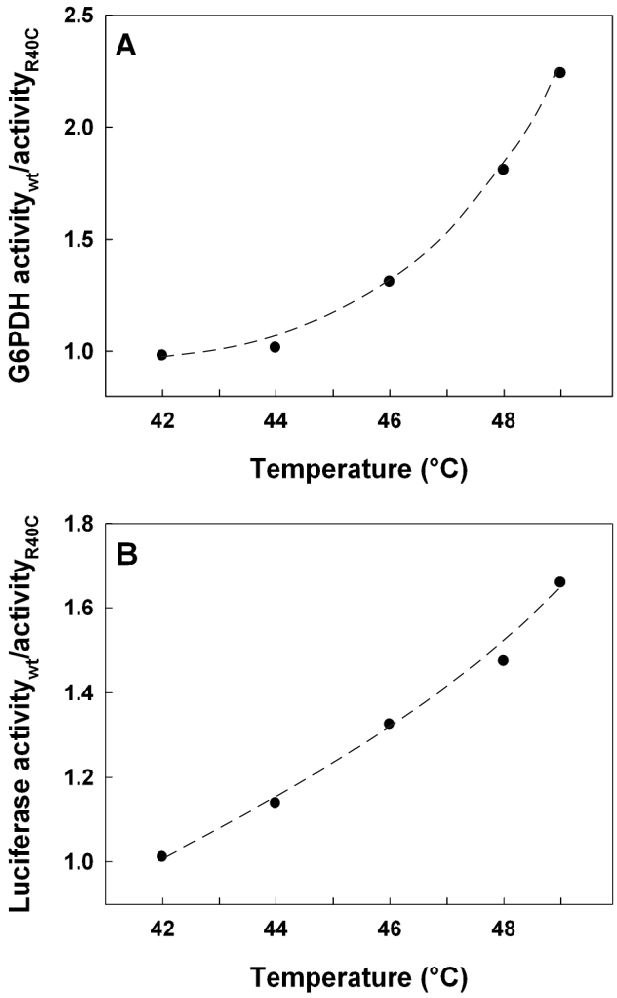

Figure 6 Efficiency of thermosensor control by GrpE as a function of temperature. (**A**) G6PDH (250 nM), 3.5 µM DnaK, 0.7 µM DnaJ, 10 mM ATP plus 3 µM wt GrpE or 3.6 µM GrpE(R40C) (the reason for using a 1.2 times higher GrpE(R40C) concentration is explained in the legend to Figure 4D) were incubated for 15 min at the indicated temperatures. After heat shock, 5 units of apyrase were added and the samples incubated at 25°C. The G6PDH activity$_{wt}$/activity$_{R40C}$ ratio after a refolding period of 60 min was plotted as a function of temperature. (**B**) An analogous experiment was performed with 250 nM luciferase.

The ADP/ATP exchange activities of wt and disulfide-stabilized GrpE thus diverge more and more with increasing temperature above 40°C. To examine whether the difference between wt GrpE and GrpE(R40C) in preventing protein aggregation during heat shock, as observed in the experiments of Figures 4B and 5B, is indeed due to their divergent nucleotide exchange activities at higher temperature, we studied their efficiency as co-chaperone in the prevention of protein aggregation as a function of temperature. G6PDH was exposed to temperatures from 42°C to 49°C for 15 min in the presence of DnaK, DnaJ, ATP plus

wt GrpE or GrpE(R40C). The spontaneous refolding of G6PDH at 25°C was then measured after addition of apyrase. After a heat shock at 42°C, the yield of G6PDH activity in the case of wt GrpE ($activity_{wt}$) was about the same as that in the presence of GrpE(R40C) ($activity_{R40C}$), resulting in an $activity_{wt}/activity_{R40C}$ ratio of ~1 (Figure 6A). With increasing temperature, however, the advantage of wt GrpE over GrpE(R40C) became increasingly evident. When luciferase was used as substrate, similar results were obtained (Figure 6B). The increase in the $activity_{wt}/activity_{R40C}$ ratio with increasing temperature appears to be linked with the differential thermal behavior of wt GrpE and GrpE(R40C) (Figure 4E). Thus, the superior performance of wt GrpE as compared with GrpE(R40C) (Figure 4 and 5) is to be ascribed to the intact thermosensor control of the chaperone system.

Effect of GrpE concentration on the retardation of luciferase heat inactivation – Is the immediate thermal adaptation of the chaperone system, i.e. the effect of the GrpE thermosensor control, also observable directly during heat shock? We followed the loss of activity of luciferase at 44°C as a function of GrpE concentration in the presence of DnaK, DnaJ and ATP. The rate of heat inactivation of luciferase was found to depend on GrpE concentration (Figure 7A). The maximum protective effect of the chaperone system was found again at a GrpE concentration of 1 μM (Figure 7B). Thus, the dose-response curve of GrpE in this experiment was similar to that obtained by measuring the amount of refolded luciferase after heat shock (see Figure 5B). Apparently, GrpE-dependent T→R shifting of the steady-state of the DnaK ATPase cycle not only affects the fraction of refolding-competent protein after heat shock but also the rate of heat inactivation of protein substrate.

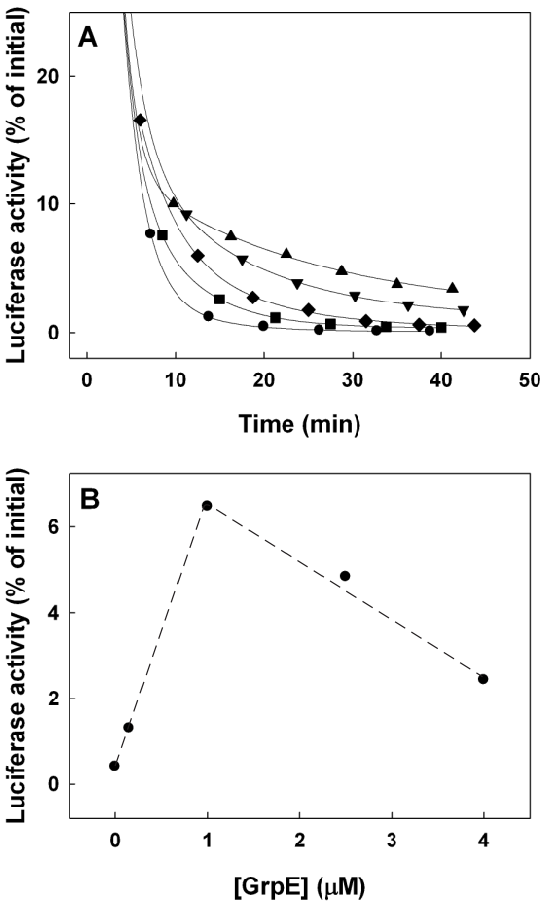

Figure 7 Effect of GrpE concentration on the retardation of luciferase heat inactivation. (**A**) Luciferase (250 nM) was incubated at 44°C in the presence of 3.5 μM DnaK, 0.7 μM DnaJ, 10 mM ATP and GrpE at the indicated concentrations: 0 (●), 0.15 (■), 1.0 (▲), 2.5 (▼), and 4.0 μM GrpE (◆). The activity of luciferase was measured as a function of time and the data points were fitted with a double-exponential equation. (**B**) Luciferase activity at 20 min as obtained by interpolation was plotted as a function of GrpE concentration.

Thermosensor effect of GrpE in the retardation of heat inactivation of luciferase – To explore whether wt GrpE with intact thermosensor retarded the heat inactivation of protein substrate to a greater extent than GrpE(R40C), we subjected luciferase to a temperature of 48°C in the presence of DnaK, DnaJ, ATP plus wt GrpE or GrpE(R40C) and monitored the loss of luciferase activity. The chosen concentrations of wt and mutant GrpE were either in the range below or above 1 μM, which is the optimum concentration for protecting luciferase against heat inactivation (see Figure 7B). After heat exposure at low GrpE concentrations, the activity of luciferase in the case of wt GrpE ($activity_{wt}$) was lower than that in the case of GrpE(R40C) ($activity_{R40C}$), the $activity_{wt}/activity_{R40C}$ ratio being 0.8 (Table I).

Table I Thermosensor effect of GrpE in the retardation of heat inactivation of luciferase. Luciferase (250 nM) was incubated at 48°C in the presence of 3.5 μM DnaK, 0.7 μM DnaJ, 10 mM ATP plus wt GrpE or GrpE(R40C). The loss in enzymic activity was measured as a function of time. Luciferase activity at 7.5 min in the presence of wt GrpE ($activity_{wt}$) or GrpE(R40C) ($activity_{R40C}$) was obtained by interpolating the data points with a double-exponential equation (compare with data in Figure 7). The GrpE concentrations were <1 μM (0.5 μM wt GrpE or 0.6 μM GrpE(R40C)) and >1 μM (4.0 μM wt GrpE or 4.8 μM GrpE(R40C)), the GrpE(R40C) concentrations being 1.2 times higher as those of wt GrpE (for explanation, see legend to Figure 5D). Averaged values and their standard deviations from 4 experiments are shown. The value of the ratio corresponds to the average of the ratios from the four data pairs.

GrpE concentration	$Activity_{wt}$	$Activity_{R40C}$	$Activity_{wt}/activity_{R40C}$ ratio
	% of initial		
Low (<1 μM)	0.106 ± 0.010	0.139 ± 0.008	0.81 ± 0.07
High (>1 μM)	0.133 ± 0.006	0.094 ± 0.006	1.43 ± 0.04

At high GrpE concentrations, wt GrpE retarded the inactivation of luciferase during heat shock to a greater extent than GrpE(R40C), the $activity_{wt}/activity_{R40C}$ ratio being 1.4. Thus, the beneficial effect of the thermosensor control of the chaperone system during heat shock is not only evident from the yield of refolding-competent protein after heat shock but also from the retardation of protein inactivation during heat shock.

Concluding remarks

The ATPase cycle of DnaK is controlled by the two co-chaperones DnaJ and GrpE, which through stimulation of ATP hydrolysis and ADP/ATP exchange promote substrate binding and release, respectively. The DnaK/DnaJ/GrpE/ATP machinery exerts two apparently dissimilar chaperone functions, i.e. the refolding of denatured proteins and the prevention of protein aggregation. Both processes depend on a continuously running chaperone cycle and seem not to be always equally important. The folding or refolding of proteins under normal growth conditions requires an optimally tuned binding and release cycle, which is primarily controlled by DnaJ. The formation of ternary (ATP·DnaK)·substrate·DnaJ complexes increases the rate of ATP hydrolysis by one to two orders of magnitude, locking ADP·DnaK onto the substrate polypeptide (Laufen et al., 1999; Han and Christen, 2003). DnaJ thus

monitors the concentration of non-native proteins and feeds them into the chaperone cycle (Han and Christen, 2003). At heat shock temperatures, however, prevention of protein aggregation through enhanced sequestering of misfolded polypeptide segments by DnaK seems to become more important: Heat-sensing GrpE (Grimshaw et al., 2001; Groemping and Reinstein, 2001; Gelinas et al., 2002) joins DnaJ in controlling the chaperone cycle; reduction of its nucleotide exchange activity results in a T→R shift of DnaK and thus in a higher fraction of substrate being sequestered by high-affinity DnaK (Siegenthaler et al., 2004). This sequestering is also a dynamic process exerted through a rapid T-R cycle of the chaperone system. In the present study, we show (i) that an optimum GrpE concentration exists for the assistance of folding/refolding as well as the prevention of heat aggregation of proteins, and (ii) that the thermosensor control of the DnaK/DnaJ/GrpE system indeed improves its chaperone effect at heat shock conditions. Desensitization of the thermosensor by stabilizing the long helix pair in the GrpE dimer with an NH_2-terminal intersubunit disulfide bond (GrpE(R40C); Figure 2) impaired the protection of protein substrates during heat shock.

Importantly, the advantageous effect of the thermosensor control by GrpE may be assumed to be actually much larger than is manifest in the experiments with GrpE(R40C) (Figure 4B, 5B and 6), because the crosslinked helices in GrpE(R40C) do not convert GrpE into a heat-stable protein with an activity that exponentially increases with temperature (see Figure 4E). Such an Arrhenius temperature dependence is for instance observed for the spontaneous nucleotide exchange in DnaK or for the DnaJ-stimulated hydrolysis of DnaK-bound ATP (Grimshaw et al., 2001). GrpE(R40C) is thus an imperfect surrogate for a nucleotide exchange factor with normal Arrhenius temperature dependence.

GrpE, in contrast to DnaJ (Langer et al., 1992; Szabo et al., 1994), does not act as a chaperone on its own. Nevertheless, it is the only member of the DnaK/DnaJ/GrpE system that is essential for bacterial growth at all temperatures (Ang and Georgopoulos, 1989),

emphasizing the importance of thermosensor control of the ATPase cycle *in vivo*. In a recent study with a GrpE-deficient strain, the expression of recombinant GrpE has been varied by varying the concentration of the inducer (Brehmer et al., 2004). Too little as well as too much GrpE reduced cellular growth, underlining again the importance of an optimally controlled ATPase cycle of DnaK. The DnaK system in *E. coli* and its homolog in *T. thermophilus* (Groemping and Reinstein, 2001) are not the only chaperone systems that take advantage of direct thermal control. Most recently, the endoplasmic reticulum-resident chaperone calreticulin was shown to undergo a thermal transition which enhances its ability to bind polypeptide substrates and improves the prevention of protein aggregation during heat shock (Rizvi et al., 2004). The occurrence in all three biological kingdoms emphasizes the importance of direct thermal control in chaperone systems.

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Tuning of DnaK chaperone action by non-native-protein sensor DnaJ and thermosensor GrpE

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Abstract

DnaK, an Hsp70 molecular chaperone, processes its substrates in an ATP-driven cycle, which is controlled by the co-chaperones DnaJ and GrpE. The kinetic analysis of substrate binding and release has as yet been limited to fluorescence-labeled peptides. Here, we report a comprehensive kinetic analysis of the chaperone action with protein substrates. The kinetic partitioning of the (ATP·DnaK)·substrate complexes between dissociation and conversion into stable (ADP·DnaK)·substrate complexes is controlled by DnaJ. This triage mechanism efficiently selects from the (ATP·DnaK)·substrate complexes those to be processed in the chaperone cycle; at 45°C, the fraction of protein complexes fed into the cycle is 20 times higher than that of peptide complexes. The thermosensor effect of the ADP/ATP exchange factor GrpE retards the release of substrate from the cycle at higher temperatures; the fraction of total DnaK in stable (ADP·DnaK)·substrate complexes is 2 times higher at 45°C than at 25°C. Monitoring the cellular situation by DnaJ as non-native-protein sensor and GrpE as thermosensor thus directly adapts the operational mode of the DnaK system to heat shock conditions.

Introduction

Molecular chaperones of the 70-kDa heat shock protein (Hsp70) family assist folding and refolding of nascent and stress-denatured proteins, assembly and disassembly of protein complexes as well as translocation of polypeptide chains across membranes. All these activities appear to rely on the transient interaction of Hsp70 with short hydrophobic segments of their protein substrates. The cycle of substrate binding and release is driven by the hydrolysis of ATP. DnaK, an Hsp70 homolog in *Escherichia coli*, consists of a 44-kDa NH_2-terminal ATPase domain (Flaherty et al., 1990; Harrison et al., 1997) and a 25-kDa COOH-terminal substrate-binding domain (Zhu et al., 1996).

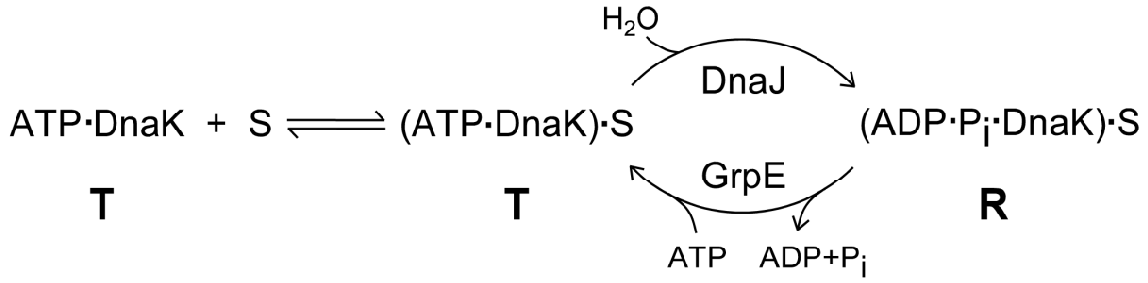

Figure 1 The DnaK/DnaJ/GrpE chaperone cycle. The substrate S first associates with ATP-liganded T-state DnaK. DnaJ then feeds the substrate into the cycle by stimulating the hydrolysis of DnaK-bound ATP. In the high-affinity R state, the substrate remains bound to DnaK, the dissociation rate being too slow to be of physiological significance. GrpE releases the substrate from the cycle by facilitating ADP/ATP exchange. The fraction of high-affinity R-state DnaK, and thus of sequestered substrate, is controlled by the conjoint action of the co-chaperones DnaJ and GrpE.

Hydrolysis of DnaK-bound ATP and ADP/ATP exchange control the functional properties of the substrate-binding domain (Jiang et al., 2005). ATP-liganded DnaK (T-state DnaK) exhibits low affinity for substrates and fast rates of binding and release, whereas ADP-liganded (R-state) DnaK is characterized by high substrate affinity and slow kinetics (Palleros et al., 1993; Schmid et al., 1994). DnaK acts in concert with two co-chaperones (Figure 1). DnaJ, an Hsp40 homolog, stimulates the hydrolysis of ATP and thus promotes the formation of high-affinity (ADP·DnaK)·substrate complexes, while GrpE facilitates the exchange of ADP for ATP and thus triggers the release of substrate from the cycle (Liberek et al., 1991; Karzai and McMacken, 1996; Laufen et al., 1999).

Fluorescence-labeled peptides have allowed the kinetic analysis of the formation of DnaK·peptide complexes (Schmid et al., 1994). With protein substrates, however, no kinetic data have been reported to date, DnaK·protein complexes having solely been examined by size-exclusion chromatography (Palleros et al., 1993), non-denaturing gel electrophoresis (Harrison et al., 1997) and by measuring fluorescence anisotropy under steady-state conditions (Chattopadhyay and Roy, 2002).

A major question in the field of molecular chaperones is how targeted chaperone action is selectively triggered. Protein substrates such as firefly luciferase, σ^{32} (Laufen et al., 1999), denatured rhodanese or RepA (Han and Christen, 2003, 2004) accelerate the

DnaJ-stimulated ATP hydrolysis by one to two orders of magnitude. DnaJ not only stimulates the ATPase activity of DnaK, but also associates with unfolded proteins (Langer et al., 1992; Gamer et al., 1996; Rüdiger et al., 2001). DnaJ has been suggested to select the chaperone substrates and then to transfer them to DnaK (Gamer et al., 1996; Laufen et al., 1999; Rüdiger et al., 2001). However, no experimental evidence has been reported as yet for this substrate transfer. A plausible mechanism of the targeting action of DnaJ is provided by the concept of a *cis*-effect of DnaJ on DnaK in ternary (ATP·DnaK)·substrate·DnaJ complexes (Han and Christen, 2003, 2004): the simultaneous binding of DnaK and DnaJ to one and the same polypeptide chain results in a higher effective concentration of DnaJ.

During heat shock and other cellular stress, the expression level of DnaK and its co-chaperones is enhanced to cope with the rising amount of misfolded and aggregation-prone proteins (for a recent review, see Wick and Egli, 2004). In *E. coli*, this well-known heat shock response is mediated by the transcription factor sigma 32 (σ^{32}), which directs RNA polymerase to transcribe the particular set of heat shock genes. Within minutes after onset of the heat shock (temperature ≥42°C), the cellular concentration of DnaK is increased about twofold (Mogk et al., 1999; Liu, 2002). Recent *in vitro* experiments with the isolated DnaK/DnaJ/GrpE chaperone system have indicated that the system also responds directly to heat-shock conditions; a mere increase in temperature suffices to increase the fraction of peptide substrate sequestered by DnaK (Siegenthaler et al., 2004). This effect is due to the differential temperature dependence of the activities of the two co-chaperones (Grimshaw et al., 2001). At heat-shock temperatures, GrpE undergoes a fully reversible conformational transition, which decreases its nucleotide exchange activity (Grimshaw et al., 2001, 2003; Groemping and Reinstein, 2001; Gelinas et al., 2002, 2003; Moro and Muga, 2006). In contrast, DnaK and DnaJ do not show any conformational changes between 15°C and 48°C.

Here, we report the kinetics of binding, release and dynamic sequestering of protein substrates in the DnaK chaperone cycle under normal and heat-shock conditions. Our results

show that the *cis*-effect of DnaJ in ternary (ATP·DnaK)·substrate·DnaJ complexes acts as a triage mechanism selectively feeding non-native proteins into the chaperone cycle. DnaJ as non-native-protein sensor and GrpE as thermosensor directly adapt the operational mode of the chaperone system to heat shock conditions.

Experimental procedures

Materials – The H541C mutation was introduced into the gene of wild-type (wt) DnaK in plasmid pTPG9 (a gift from Dr. C. Georgopoulos and Dr. D. Ang, Geneva) with the QuikChange site-directed mutagenesis kit from Stratagene, La Jolla, CA, USA. The nucleotide replacements were verified by DNA sequence analysis. Wt DnaK and DnaK H541C were purified as described previously (Feifel et al., 1996). The concentration of wt DnaK was determined photometrically with the molar absorption coefficient ε_{280} = 14,500 $M^{-1}cm^{-1}$ (Hellebust et al., 1990). The stock solution of wt DnaK was stored in assay buffer (25 mM Hepes/NaOH, 100 mM KCl, 10 mM $MgCl_2$, pH 7.4) at –80°C. For fluorescence-labeling, 760 µM DnaK H541C in 25 mM Hepes/NaOH, 100 mM KCl, 10 mM $MgCl_2$, pH 7.0 was reacted with a 5-fold molar excess of acrylodan in the presence of 10 mM ATP (to prevent labeling at Cys15 located in the ATPase domain) for 3 h at room temperature. The acryl group of acrylodan served to attach the environmentally sensitive fluorophore covalently to the sulfhydryl group of Cys541. The reaction mixture was filtrated (0.2 µm); acrylodan-labeled DnaK H541C (a-DnaK H541C) was purified by size-exclusion chromatography (Fractogel EMD BioSec 650 S from Merck) in 25 mM Tris/HCl, 1 mM EDTA, pH 8.0 containing 10 mM 2-mercaptoethanol, transferred into assay buffer with a Sephadex G-25 PD-10 column (Amersham Biosciences, UK), and stored at –80°C. Incorporation of a single fluorophore per DnaK H541C molecule was confirmed by mass spectrometry. The concentration of the stock solution was determined with the Bradford

method (Bradford, 1976) with bovine serum albumin (BSA) as standard. DnaJ and GrpE (gifts from Dr. H.-J. Schönfeld, Hoffmann-La Roche, Basel) were prepared as reported elsewhere (Schönfeld et al., 1995a; Schönfeld et al., 1995b). The stock solutions in 50 mM Tris/HCl, 100 mM NaCl, pH 7.7 were kept at –80°C. The concentrations of DnaJ and GrpE were determined photometrically with ε_{277} = 18,100 $M^{-1}cm^{-1}$ and ε_{279} = 2,720 $M^{-1}cm^{-1}$, respectively. Throughout this report, the indicated protein concentrations refer to the respective protomer. Bacteriophage P1 RepA (the expression strain was a gift from Dr. D. Chattoraj, National Cancer Institute, Bethesda, MD, USA) was expressed and purified as described (Abeles, 1986). The concentration of the stock solution in assay buffer was determined with the Bradford method with BSA as standard. Bovine α-lactalbumin (type III) was purchased from Sigma. For permanent denaturation, α-lactalbumin (850 μM) was dissolved in 6 M guanidine hydrochloride, 0.5 M Tris/HCl, 5 mM EDTA, pH 8.6 containing 10 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Pierce, Rockford, IL, USA) as reducing agent and incubated for 1 h at 37°C. The sulfhydryl groups of the 8 cysteine residues were reacted with 500 mM iodoacetamide (Sigma) for 5 min at 25°C. The reaction was stopped with 1 M 2-mercaptoethanol and the denatured protein was transferred into 3 M guanidine hydrochloride, 20 mM Tris/HCl, pH 7.4 with a PD-10 column and stored at –80°C. The molecular mass of reduced and carboxymethylated α-lactalbumin (RCMLA) was confirmed by mass spectrometry and the concentration (420 μM) of the stock solution was determined with the Bradford method with BSA as standard. Peptides pp (CALLQSRLLLSAPRRAAATARA) and p4 (CALLQSRLLS), both derivatives of the prepiece of chicken mitochondrial aspartate aminotransferase, were custom-synthesized by Chiron, Australia, or synthesized by Dr. S. Klauser in our Institute with an ABI 430A Peptide Synthesizer (Applied Biosystems). The concentration of the stock solution of pp in 1 mM dithiothreitol was determined by amino acid analysis. Peptide p4 was acrylodan-labeled and

purified as described (Siegenthaler et al., 2004). The concentration of the a-p4 stock solution in 30% (v/v) acetonitrile was determined photometrically with $\varepsilon = 20{,}000\ M^{-1}cm^{-1}$ (Molecular Probes). ATP-Na_2 (purity >98%) and ADP-Na_2 (purity >90%) were purchased from Fluka. *N*8-(4-*N*'-methylanthraniloylaminobutyl)-8-aminoadenosine-5'-diphosphate (MABA-ADP; a gift from Dr. J. Reinstein, Max Planck Institut für medizinische Forschung, Heidelberg, Germany) had been synthesized as described (Theyssen et al., 1996). [2,5',8-^{3}H]Adenosine-5'-triphosphate ammonium salt (37.0 Ci/mmol) was from Amersham Biosciences.

Luciferase refolding assay – *Photinus pyralis* luciferase (Roche) was denatured in 6 M guanidine hydrochloride, 50 mM Tris/HCl, 50 mM KCl, 15 mM $MgCl_2$, pH 7.5 containing 0.05 mg/ml BSA for 30 min at 25°C. For refolding, the luciferase solution was prediluted into assay buffer containing DnaJ, GrpE plus ATP, added (70 µl) to test tubes on ice containing a-DnaK H541C or wt DnaK at different concentrations in 30 µl, and incubated at 25°C. In the refolding solution, the concentration of luciferase and guanidine hydrochloride was 250 nM and 62.5 mM, respectively. Luciferase activity was measured in a Lumac Biocounter M1500 as described previously (Bischofberger et al., 2003).

Circular dichroism measurements – Circular dichroism was measured with a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan) in a thermostatically controlled cuvette of 0.2 mm path length. Spectra of 15 µM a-DnaK H541C or wt DnaK in 25 mM potassium phosphate, 150 µM ADP, 150 µM $MgCl_2$, pH 7.0 were recorded from 250 nm to 195 nm (bandpass 1 nm) at 25°C.

Equilibrium fluorescence measurements – A Perkin-Elmer spectrofluorimeter LS50B, equipped with a stirrer and a thermostated cuvette holder, was used to record fluorescence

emission spectra of a-DnaK H541C. The excitation wavelength was set at 370 nm (bandpass 4 nm) and the spectra were recorded from 400 nm to 600 nm (bandpass 4 nm). The dissociation equilibrium constants (K_d) of a-DnaK H541C for RepA, RCMLA or pp were determined by fluorescence titration. The K_d values were obtained from least-squares fits of the fluorescence emission intensity of a-DnaK H541C at 500 nm (F) as a function of substrate concentration to the equation

$$F = F_0 + PL\cdot(F_{max}-F_0)/P_t = F_0 + [(F_{max}-F_0)/(2P_t)][(K_d+L_t+P_t) - ((K_d+L_t+P_t)^2 - 4P_t\cdot L_t)^{0.5}].$$

PL denotes the concentration of a-DnaK H541C·substrate complex, P_t the total concentration of a-DnaK H541C, L_t the total substrate concentration, F_0 the emission intensity of a-DnaK H541C in the absence of substrate, and F_{max} the emission intensity in the presence of substrate at saturating concentrations. All experiments were performed in assay buffer at a volume of 800 µl in a 1 × 0.4 cm cuvette. Fluorescence intensity and concentration of substrate were linearly corrected for the increasing sample volume during titration.

Fast kinetic fluorescence measurements – A stopped-flow apparatus from Applied Photophysics SX18 MV served to record changes in the fluorescence emission intensity of a-DnaK H541C, a-p4 and MABA-ADP. a-DnaK H541C and a-p4 were excited at 370 nm (bandpass 4.5 nm) and MABA-ADP was excited at 360 nm (bandpass 5.0 nm). The emitted light passed through a high-pass filter with a 455-nm cut-off. The temperature of the syringes and the cuvette was controlled with a circulating water bath (± 0.5°C). The solutions were equilibrated for at least 3 min at the respective temperature before starting the reaction by mixing 70 µl of each solution. All experiments were performed in assay buffer. Reaction traces of at least four measurements were averaged. The second-order binding rate constants (k_1) and the dissociation rate constants (k_{-1}) of a-DnaK H541C for RepA, RCMLA or pp were obtained from least-squares fits of the observed pseudo-first order rate constant of complex

formation (k_{obs}) as a function of substrate concentration to the equation $k_{obs} = k_1 \cdot [substrate] + k_{-1}$. The dissociation constant of the first step in complex formation (K_{-1}) was calculated as $K_{-1} = k_{-1}/k_1$. K_{-1} should not be mistaken for the overall dissociation equilibrium constant K_d which was determined by fluorescence titration.

Single-turnover ATPase activity – To preform ATP·DnaK complexes, DnaK was incubated with excess radioactively labeled ATP for 10 min at 4°C. DnaK-bound ATP was separated from free ATP by rapid size-exclusion chromatography with a Sephadex G-50 NICK column (Amersham Biosciences, UK) in assay buffer at 4°C as described (McCarty et al., 1995). DnaJ was preincubated in assay buffer in the absence and presence of RepA, RCMLA or pp for 3 min at the indicated temperatures, and ATP hydrolysis started by addition of ATP·DnaK complex to a final volume of 50 µl. The reaction was quenched at certain time points by mixing 4 µl of the sample solution with 4 µl formic acid. ADP was separated from ATP on poly(ethyleneimine)-cellulose thin-layer plates (Merck) with 1 M formic acid / 0.7 M lithium chloride as eluant. ATP and ADP were quantified by liquid-scintillation counting. A least-squares fit of the increase in ADP concentration with time to a single-exponential equation gave the rate constant of ATP hydrolysis.

Steady-state ATPase activity – Test tubes containing DnaK, DnaJ and GrpE in assay buffer were preincubated in the absence and presence of RepA, RCMLA or pp for 3 min at the indicated temperature. ATP hydrolysis was started by addition of 10 µM (or 100 µM in the case of RepA) radioactively labeled ATP to a final volume of 50 µl. The production of ADP was followed as described above. In all assays, the fraction of ATP hydrolyzed did not exceed 20% of the initial. The extent of spontaneous ATP hydrolysis during the assay was negligible.

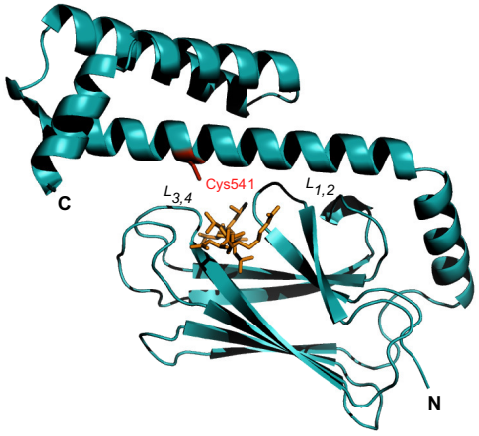

Figure 2 Structure of the DnaK substrate-binding domain. The truncated substrate-binding domain of DnaK (residues 389-607, Δ608-638) has been co-crystallized with peptide NR (NRLLLTG; orange sticks) (Zhu et al., 1996). The substrate binding cleft is primarily formed by the two loops $L_{1,2}$ and $L_{3,4}$ of the β-sandwich subdomain. The substrate peptide is unidirectionally bound in an extended conformation (see also Landry et al., 1992) and interacts with DnaK through numerous van der Waals contacts of its main chain and side chains as well as seven main-chain hydrogen bonds. The α-helical segments do not directly participate in the binding of the peptide; they appear to serve as a flexible lid over the substrate-binding cavity. The lid is supposed to be predominantly in an either opened or closed conformation in ATP-liganded and ADP-liganded (or nucleotide-free) DnaK, respectively. Residue Cys541 (shown in red), to which the fluorescent reporter group acrylodan was attached, faces the peptide-binding site. The atomic coordinates are from Protein data bank entry 1DKX (Zhu et al., 1996).

Results

We determined the kinetics of the following phases in the DnaK/DnaJ/GrpE chaperone cycle (Figure 1): (1) binding and release of protein substrates to and from ATP·DnaK by real-time recording of changes in the fluorescence of acrylodan-labeled DnaK H541C, (2) T→R conversion including the *cis*-effect of DnaJ on the (ATP·DnaK)·protein complexes and (3) R→T conversion including the thermosensor effect of GrpE. All assays were run at 25°C, 37°C and 45°C. The concentrations of the chaperones were set at 500 nM DnaK, 100 nM DnaJ, and 200 nM GrpE in accord with the estimated concentration ratios of DnaK:DnaJ:GrpE = 5:1:2 in the cell (Diamant and Goloubinoff, 1998; Liu, 2002).

Fluorescence-labeled a-DnaK H541C – To label DnaK with the thiol-reactive, environmentally sensitive fluorophore acrylodan, we introduced an additional cysteine residue in DnaK by amino acid substitution.

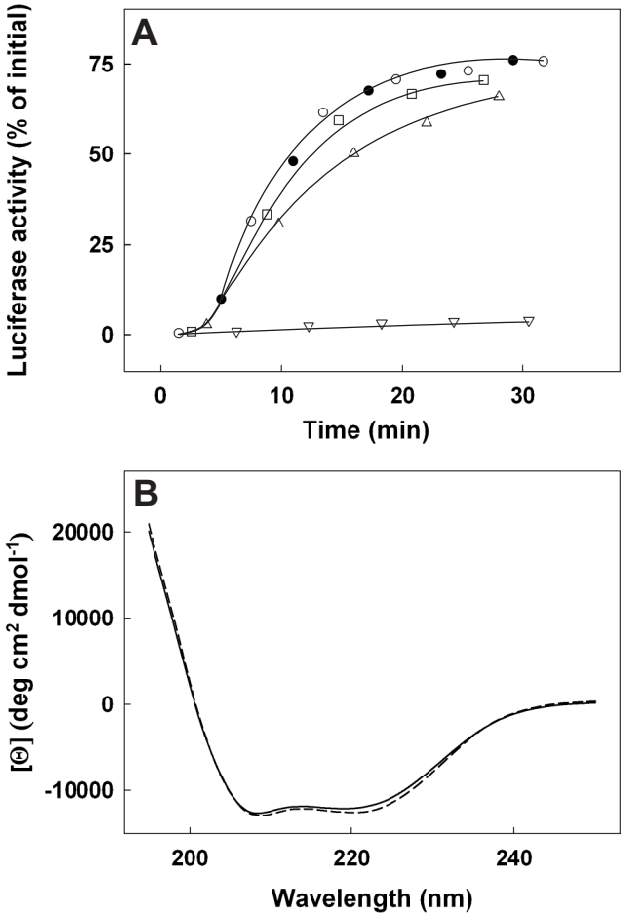

Figure 3 Refolding activity and circular dichroism spectrum of a-DnaK H541C. (**A**) Chemically denatured luciferase (250 nM; for details, see Experimental procedures) was refolded at 25°C in the presence of 0.7 µM DnaJ, 0.35 µM GrpE, 10 mM ATP plus a-DnaK H541C or wt DnaK at the indicated concentrations: 3.5 µM a-DnaK H541C (●), 3.5 µM wt DnaK (○), 2.8 µM wt DnaK (□), and 2.1 µM wt DnaK (△); control without DnaK (▽). The measurements at 2.8 µM and 2.1 µM wt DnaK (corresponding to 80% and 60% of 3.5 µM wt DnaK, respectively) serve to calibrate the chaperone efficacy scale. Enzymic activity of refolding luciferase was measured at the indicated time points. The lines have been added as a guide to the eye. (**B**) Circular dichroism of 15 µM a-DnaK H541C or wt DnaK was recorded in 25 mM potassium phosphate, 150 µM ADP, 150 µM $MgCl_2$, pH 7.0 at 25°C. Spectra of a-DnaK H541C and wt DnaK are indicated by solid and dashed lines, respectively.

Because DnaK has only one cysteine residue, which is located in its ATPase domain and is easily blocked with ATP (Feifel, 1998), any new cysteine residue, if in accessible position, can be specifically labeled. We tested several positions, i.e. His541 (Feifel, 1998), Met469 (Tanaka et al., 2002), Glu430 and Asn463, that are located near the substrate-binding cavity of DnaK for attaching the fluorescent probe. Based on the criteria of fluorescence signal quality, maintained ATPase activity and chaperone efficacy in the luciferase refolding assay, we chose a-DnaK H541C for experimentation (Figure 2). The molecular activity of a-DnaK

H541C (500 nM) was the same as that of wt DnaK for ATP hydrolysis (k'_{cat} = 0.008 s^{-1}) in the presence of 100 nM DnaJ and 200 nM GrpE under steady-state conditions at 25°C. In the luciferase refolding assay, the same rate and yield was obtained with a-DnaK H541C as with wt DnaK (Figure 3A). Moreover, the far-UV circular dichroism spectra of a-DnaK H541C and wt DnaK were almost identical (Figure 3B).

We compared the complex formation of fluorescence-labeled a-DnaK H541C and peptide pp with the complex formation of wt DnaK and fluorescence-labeled peptide a-pp. The K_d values of a-DnaK H541C for pp in the presence of ATP or ADP were determined by fluorescence titration (Figure 4A). Binding of pp to a-DnaK H541C resulted in an increase in fluorescence emission intensity and a blue-shift in the wavelength of maximum emission. The K_d values calculated from the hyperbolic titration curves (Figure 4B) were close to the previously determined values of wt DnaK for a-pp (Table I). The second-order binding rate constant (k_1) and the dissociation rate constant (k_{-1}) of ATP·a-DnaK H541C for pp were determined by measuring the observed pseudo-first order rate constant of complex formation (k_{obs}) as a function of pp concentration (Figure 4C). The progress curves were best described with a double-exponential equation. The k_{obs} value of the first phase (amplitude ~80% of total) was linearly proportional to the pp concentration (Figure 4D). The derived values of k_1 and k_{-1} were of the same order of magnitude as those of wt DnaK for a-pp (Table I).

Table I Rate constants (k_1 and k_{-1}) and dissociation equilibrium constants for a-DnaK H541C·pp and wt DnaK·a-pp complexes in the presence of ATP at 25°C

Complex	k_1 (+ATP)	k_{-1} (+ATP)	K_{-1} (+ATP)	K_d (+ATP)	K_d (+ADP)
	$M^{-1}s^{-1}$	s^{-1}	μM	μM	μM
a-DnaK H541C·pp	880,000[a]	1.2[a]	1.4[a]	1.3[b]	0.09[c]
Wt DnaK·a-pp[d]	450,000	1.8	4.0	2.2	0.06

[a] $K_{-1} = k_{-1}/k_1$; calculated from the data of Figure 4D.
[b] Determined by fluorescence titration (Figure 4B).
[c] Determined by fluorescence titration of 100 nM a-DnaK H541C with pp (0.025 – 1.0 μM) in the presence of 1 mM ADP (for details, see Experimental procedures).
[d] From Schmid et al., 1994.

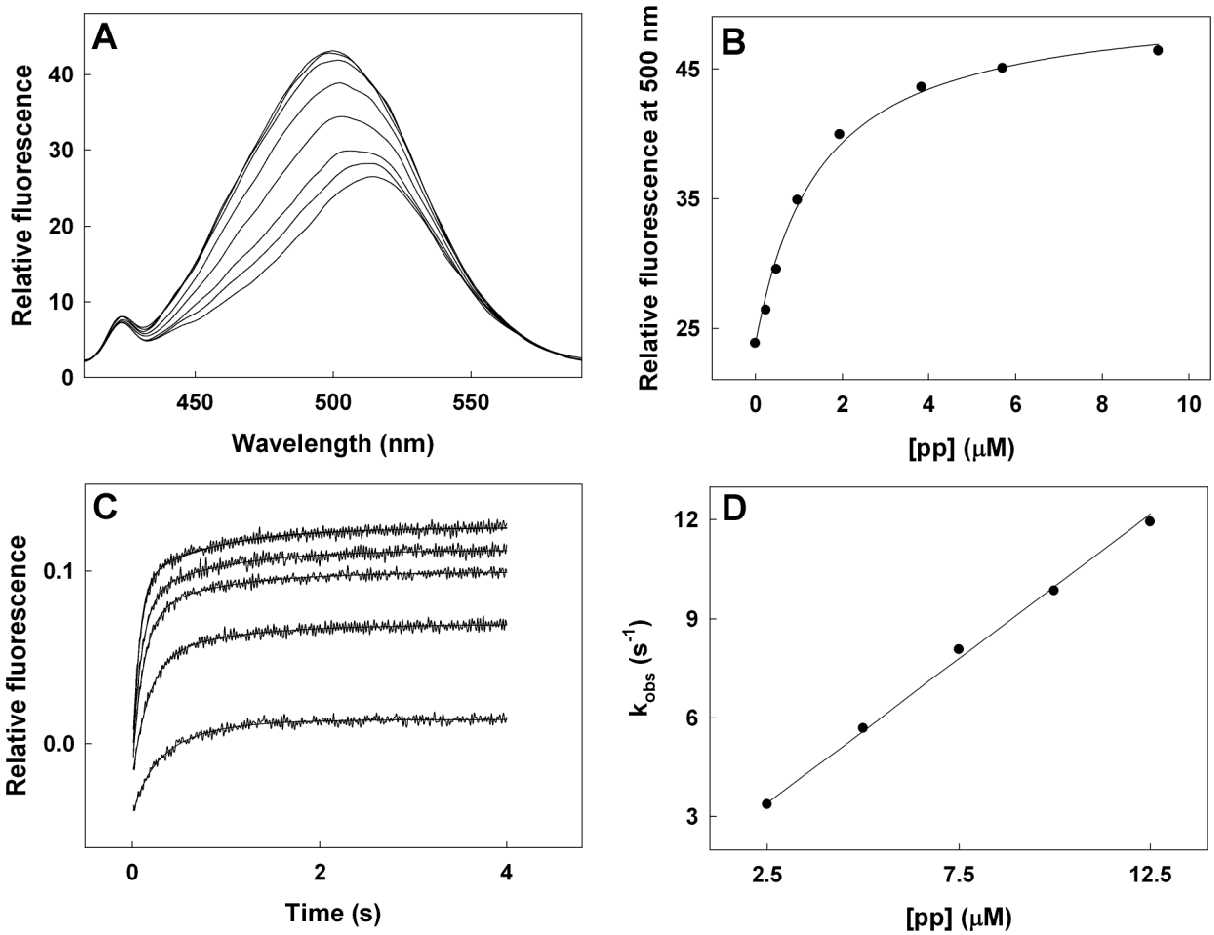

Figure 4 Complex formation of a-DnaK H541C with peptide pp. (**A**) Fluorescence titration of ATP·a-DnaK H541C with peptide pp. Fluorescence emission spectra of 200 nM a-DnaK H541C were recorded in the presence of 5 mM ATP and increasing concentrations of peptide pp (from bottom to top: 0, 0.25, 0.49, 0.98, 1.9, 3.8, 5.7, 9.3 μM) at 25°C. (**B**) Emission intensities at 500 nm of the spectra shown in Panel A were plotted against pp concentration. The data points were fitted with a hyperbolic equation (for details, see Experimental procedures). The K_d value calculated from these data is given in Table I. (**C**) Kinetics of a-DnaK H541C·pp complex formation. The following solutions were mixed in a stopped-flow device at 25°C: [500 nM a-DnaK H541C, 5 mM ATP] + [pp, 5 mM ATP]. The concentrations of pp (from bottom to top) were: 2.5, 5, 7.5, 10, 12.5 μM. The reaction traces were fitted with a double-exponential equation. (**D**) The k_{obs} values of the first phase (~80% of total amplitude) of the reaction traces shown in Panel C were plotted against the concentration of pp and the data points fitted with a linear equation (for details, see Experimental procedures). The values of k_1 and k_{-1} calculated from these data are given in Table I.

The k_{obs} value of the second, ~10 times slower phase of complex formation (amplitude ~20% of total) increased hyperbolically with increasing concentration of pp (not shown), suggesting that the rapid formation of an encounter complex is followed by a slower isomerization step. The similarity of a-DnaK H541C with wt DnaK regarding ATPase activity, performance in the luciferase refolding assay and far-UV circular dichroism spectra as well as the similar values of the binding parameters of a-DnaK H541C for pp and wt DnaK for a-pp qualify

a-DnaK H541C as a suitable means for investigating the interaction of DnaK with protein substrates. For monitoring the T→R and R→T conversions, however, a-DnaK H541C proved unsatisfactory because the difference in the fluorescence signal between the two states under the chosen conditions was too small for reliable measurements.

Complex formation of a-DnaK H541C with protein substrates – DnaK preferentially recognizes peptide segments comprising a hydrophobic core of four to five apolar amino acid residues and flanking regions with basic residues (Rüdiger et al., 1997). Such protein segments typically reside in the interior of folded proteins and are only exposed in nascent or denatured proteins. Hence, it is difficult to find proteins that are substrates for DnaK under non-denaturing conditions yet do not tend to aggregate. RepA as well as reduced and carboxymethylated α-lactalbumin (RCMLA) were found to fulfill these criteria. RepA, an initiator protein of bacteriophage P1 plasmid replication of 32 kDa, binds to DnaK in its native state (Wickner et al., 1991). RCMLA (14 kDa) is a permanently denatured but soluble substrate for DnaK (Palleros et al., 1991). We followed complex formation of ATP·a-DnaK H541C with RepA, RCMLA and, for comparison, with peptide pp at 25°C, 37°C and 45°C. The k_1, k_{-1}, K_{-1} and K_d values for the proteins RepA and RCMLA were at all temperatures found to be of the same order of magnitude as the values for peptide pp (Table II). The k_1 and k_{-1} values for RepA and RCMLA followed an Arrhenius temperature dependence between 25°C and 45°C as found for pp, indicating that neither of these proteins undergoes major structural changes between 25°C and 45°C. The K_{-1} values of RepA, RCMLA and pp did not significantly change between 25°C and 45°C as described previously for the K_d values of fluorescence-labeled NR peptide (Siegenthaler et al., 2004).

Table II Rate constants (k_1 and k_{-1}) and dissociation equilibrium constants for a-DnaK H541C·substrate complexes in the presence of ATP at different temperatures.

	25°C	37°C	45°C
Substrate	k_1 in the presence of ATP[a]		
		$M^{-1}s^{-1}$	
RepA	160,000	380,000	640,000
RCMLA	120,000	280,000	530,000
pp[d]	880,000	1,950,000	3,250,000
	k_{-1} in the presence of ATP[a]		
		s^{-1}	
RepA	4.0	7.0	8.2
RCMLA	0.9	3.7	7.7
pp[d]	1.2	6.3	12.1
	K_{-1} in the presence of ATP[a]		
		µM	
RepA	24.9	18.3	12.8
RCMLA	7.7	13.1	14.7
pp[d]	1.4	3.2	3.7
	K_d in the presence of ATP[b]		
		µM	
RepA	4.2		
RCMLA	5.5		
pp[d]	1.3		
	K_d in the presence of ADP[c]		
		µM	
RepA	0.85		
RCMLA	0.42		
pp[d]	0.09		

[a]Determined by mixing the following solutions in a stopped-flow apparatus: [500 nM a-DnaK H541C, 5 mM ATP] + [substrate, 5 mM ATP]. The concentration of substrate was varied between 2.5 µM and 12.5 µM. The reaction traces (Figure 4C shows an example) were fitted with a double-exponential equation and the observed rate constant (k_{obs}) of the first phase was used to determine the values of k_1, k_{-1} and $K_{-1} = k_{-1}/k_1$ (for details, see Experimental procedures). The approximate calculated amplitudes of the first phase with RepA, RCMLA and pp were at 25°C: 55%, 80% and 80% of the total amplitude, respectively; at 37°C: 40%, 60% and 75%, respectively; and at 45°C: 30%, 45% and 70%, respectively.

[b]Determined by fluorescence titration of 200 nM a-DnaK H541C with substrate (0.25 – 30 µM) in the presence of 5 mM ATP. The titration curve with pp is shown as an example in Figure 4A (for details, see Experimental procedures).

[c]For comparison, K_d values of (ADP·a-DnaK H541C)·substrate complexes are listed. The values were determined by fluorescence titration of 100 nM or 200 nM a-DnaK H541C with substrate (0.025 – 5 µM) in the presence of 1 mM ADP (for details, see Experimental procedures).

[d]From Table I.

DnaJ-controlled T→R conversion – The *cis*-effect of DnaJ on DnaK in ternary (ATP·DnaK)·substrate·DnaJ complexes accelerates ATP hydrolysis by one to two orders of magnitude (see Introduction). We measured the DnaJ-stimulated ATPase activity of DnaK in the presence of RepA, RCMLA or pp at 25°C, 37°C and 45°C. RepA appears to possess a DnaK-binding site and a separate binding site for DnaJ (Kim et al., 2002), while RCMLA binds only to DnaK (Palleros et al., 1993), its affinity for DnaJ being very low (Langer et al., 1992). Peptide pp binds to both chaperones (Pierpaoli et al., 1998) but is too short for forming ternary complexes. At both 37°C and 45°C, 2.5 μM and 5 μM RepA gave rise to the *cis*-effect of DnaJ and accelerated the rate of the DnaJ-stimulated ATP hydrolysis ~8 and ~15 times, respectively, under single-turnover conditions (Figure 5A, Table III), i.e. by the same factor as found earlier at 25°C (Han and Christen, 2004). In contrast, RCMLA and pp, which are substrates that do not form ternary complexes and consequently do not elicit the *cis*-effect of DnaJ, only barely stimulated the rate of ATP hydrolysis at all temperatures tested.

We determined the rate of DnaJ-stimulated T→R conversion also by measuring steady-state ATPase activity. To ensure that the release of ADP and inorganic phosphate were not rate-limiting in the overall cycle, GrpE was added in large excess. The rates determined under steady-state conditions, both in the presence and absence of RepA, proved to be similar to the single-turnover rates; in particular, the *cis*-effect of DnaJ found under single-turnover conditions is also observed in the steady state of the ATPase cycle. In the temperature range from 25°C to 45°C, the rate of ATP hydrolysis followed an Arrhenius temperature dependence (Figure 5B). Likewise, though on a lower level due to the missing *cis*-effect of DnaJ, the rate of ATP hydrolysis in the presence of RCMLA and pp as well as in the absence of substrate increased exponentially (Figure 5A, Table III). The rate of ATP hydrolysis at 25°C was linearly dependent on the concentration of DnaJ from 0.1 μM to 1 μM measured by both single-turnover and steady-state ATPase activity assays (not shown).

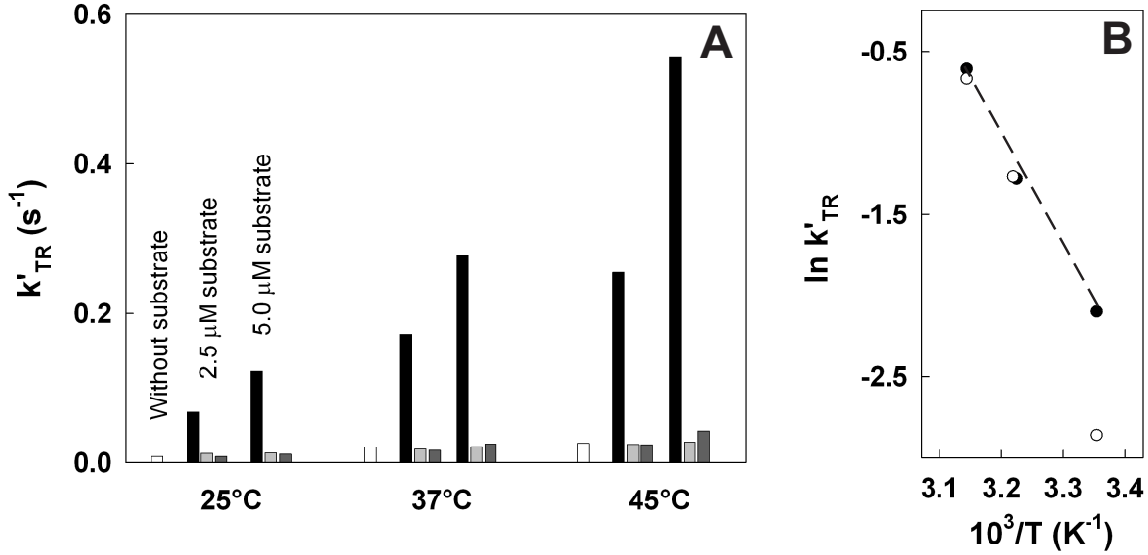

Figure 5 Rate of DnaJ-stimulated T→R conversion. (**A**) The apparent rate constant k'_{TR} of the DnaJ-stimulated hydrolysis of DnaK-bound ATP was measured under single-turnover conditions with 500 nM DnaK, 100 nM DnaJ and 500 nM ATP in the absence or presence of 2.5 µM and 5 µM RepA, RCMLA or pp at the indicated temperatures (for details, see Experimental procedures). The k'_{TR} values are displayed by columns: without substrate (white), with RepA (black), with RCMLA (light gray) and with pp (dark gray). For the numerical values, see Table III. (**B**) Arrhenius plots of the rate of ATP hydrolysis in the presence of 5 µM RepA determined by single-turnover assays (●; data from A) and, for comparison, determined by steady-state ATPase assays (○; see Experimental procedures). Steady-state ATPase activity was measured with 500 nM DnaK, 100 nM DnaJ, 5 µM RepA, 10 µM GrpE, and 100 µM ATP at the indicated temperatures. The steady-state ATPase activity measured with 20 µM DnaJ under otherwise identical conditions was found to be 0.9 s^{-1} at 25°C, indicating that the rate of the GrpE-facilitated ADP/ATP exchange at 10 µM GrpE in the assay was not less than 0.9 s^{-1} and thus fast enough for accurate determination of the rate of the DnaJ-stimulated T→R conversion. The numerical values are given in Table III. The rate in the presence of RepA at 25°C determined under steady-state conditions, for unexplained reason, was found to be ~2 times slower than the rate determined under single-turnover conditions.

Triage of (ATP·DnaK)·substrate complexes: entering the chaperone cycle versus dissociation – Upon binding of substrate to ATP-liganded DnaK, the substrate either enters the chaperone cycle through DnaJ-stimulated T→R conversion of the complex or dissociates from DnaK into the solvent (Figure 1). The fraction of substrate which is processed in the cycle is thus determined by the relative rates of these two alternative reactions. The *cis*-effect of DnaJ resulted in a higher fraction of RepA fed into the chaperone cycle compared to the corresponding fractions of RCMLA and pp (Table IV). An increase in RepA concentration from 2.5 µM to 5 µM increased the fraction about twofold at all temperatures measured.

Table III Rate constants of DnaJ-stimulated T→R conversion and GrpE-facilitated R→T conversion.

	25°C	37°C	45°C
Substrate	T→R conversion, k'_{TR} (single-turnover)[a]		
		(s^{-1})	
RepA (5 μM)	0.122	0.277	0.544
RCMLA (5 μM)	0.013	0.021	0.027
pp (5 μM)	0.011	0.024	0.042
None	0.008	0.020	0.026
	T→R conversion, k'_{TR} (steady-state)[b]		
		(s^{-1})	
RepA (5 μM)	0.057	0.280	0.512
None	0.005	0.014	0.030
	R→T conversion, k'_{RT} (without phosphate)[c]		
		(s^{-1})	
RepA (5 μM)	0.863	1.450	1.280
RCMLA (5 μM)	1.061	1.800	1.426
pp (5 μM)	0.725	1.363	1.448
None	0.750	1.331	1.174
	R→T conversion, k'_{RT} (with phosphate)[d]		
		(s^{-1})	
a-p4	0.154	0.247	0.176

[a]Determined by single-turnover ATPase activity measurements as described in Figure 5A (for details, see Experimental procedures).
[b]Determined by steady-state ATPase activity measurements at 500 nM DnaK, 100 nM DnaJ, 10 μM GrpE and 10 μM or 100 μM ATP in the absence and presence, respectively, of 5 μM RepA.
[c]Determined by fluorescence measurements with MABA-ADP as described in Figure 6A.
[d]The GrpE-facilitated rate of the R→T conversion was determined by mixing the following solutions in a stopped-flow device: [500 nM DnaK, 4 μM ADP, 200 nM a-p4, 25 mM phosphate] + [200 nM GrpE, 5 mM ATP] (for details, see Experimental procedures).

With temperature increasing from 25°C to 37°C and 45°C, an increasingly higher fraction of RepA was fed into the chaperone cycle due to the differential temperature dependence of the rate of the T→R conversion (Figure 5A, Table III) and the rate of dissociation of the (ATP·DnaK)·RepA complex (Table II). In contrast, the fractions of RCMLA and pp decreased with increasing temperature from 25°C to 45°C, due to the steep temperature dependence of their dissociation rates (Table II).

Table IV Fraction of substrate fed into the DnaK chaperone cycle. The fraction F of (ATP·DnaK)·substrate complexes, which is converted into high-affinity (ADP·DnaK)·substrate complexes, was calculated from the rate k'_{TR} of the DnaJ-stimulated T→R conversion and the dissociation rate k_{-1} of the (ATP·DnaK)·substrate complex: $F = k'_{TR}/(k'_{TR} + k_{-1})$. The k'_{TR} values, determined by single-turnover ATPase activity measurements, and the k_{-1} values, were taken from Figure 5A and Table II, respectively.

	25°C	37°C	45°C
Substrate	Fraction of (ATP·DnaK)·substrate complex entering the cycle		
	% of total		
RepA (5 μM)	3.0	3.8	6.2
RepA (2.5 μM)	1.7	2.4	3.0
RCMLA (5 μM)	1.4	0.6	0.4
pp (5 μM)	0.9	0.4	0.3

At 45°C and 5 μM RepA, the fraction of RepA fed into the cycle was more than one order of magnitude higher than that of RCMLA or pp (Table IV).

GrpE-controlled R→T conversion − The ADP/ATP exchange factor GrpE acts as thermosensor for the chaperone system (see Introduction). We measured the GrpE-facilitated release of fluorescence-labeled MABA-ADP (Theyssen et al., 1996) in the presence of RepA, RCMLA or pp at 25°C, 37°C and 45°C. The rate of MABA-ADP release proved to be substrate-independent at all temperatures (Figure 6A, Table III). However, the measurement of the release of MABA-ADP does not include the complete R→T conversion, we therefore determined the rate of the R→T conversion also by the release of acrylodan-labeled peptide p4 (a-p4) upon conversion of high-affinity (ADP·DnaK)·a-p4 into low-affinity (ATP·DnaK)·a-p4 complex. The experiments were performed in the presence of saturating concentrations of inorganic phosphate, because the release of ADP from DnaK is markedly slowed down by inorganic phosphate (Theyssen et al., 1996; Russell et al., 1998). The rate of the GrpE-facilitated release of a-p4, which was 1.41 s^{-1} at 25°C in the absence of inorganic phosphate, was indeed decelerated to 0.15 s^{-1} in the presence of phosphate.

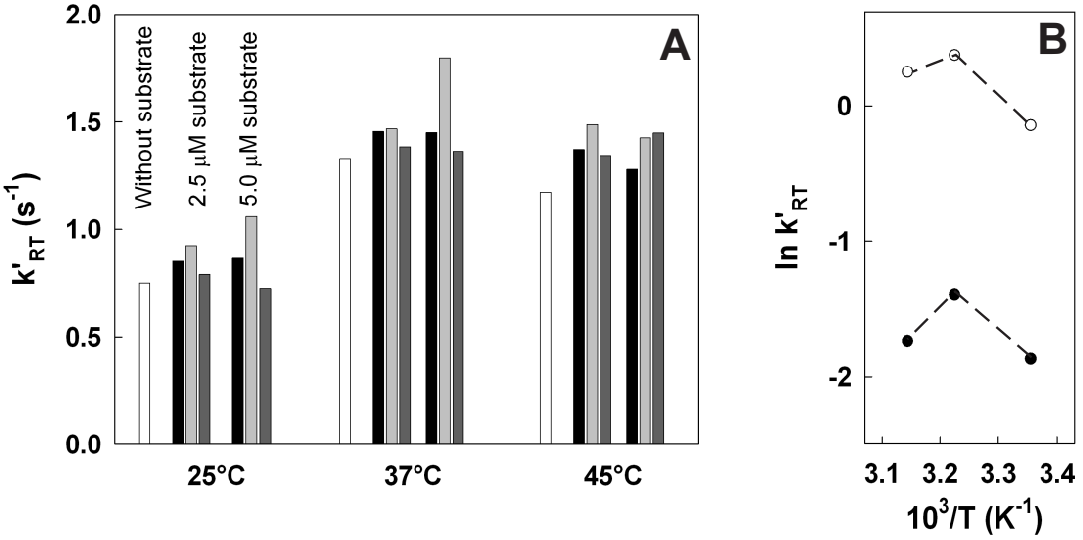

Figure 6 Rate of GrpE-facilitated R→T conversion. (**A**) The apparent rate constant k'_{RT} of the GrpE-facilitated ADP/ATP exchange was measured by mixing the following solutions in a stopped-flow device at the indicated temperatures: [500 nM DnaK, 500 nM ADP, 500 nM MABA-ADP, substrate] + [200 nM GrpE, 5 mM ATP]. The concentrations of substrate (RepA, RCMLA or pp) were 0, 2.5, and 5 μM. Before mixing, the solutions were incubated for at least 2.5 h at 4°C to preform (MABA-ADP·DnaK)·substrate complexes. The decrease in fluorescence emission of MABA-ADP upon release from DnaK was recorded and the reaction traces were fitted with a single-exponential equation (for details, see Experimental procedures). The k'_{RT} values are displayed by columns: without substrate (white), with RepA (black), with RCMLA (light gray) and with pp (dark gray). For the numerical values, see Table III. (**B**) Arrhenius plots of the nucleotide exchange rate in the presence of 5 μM RepA determined by the release of MABA-ADP in the absence of inorganic phosphate (○; data from A) and determined by the release of a-p4 in the presence of 25 mM inorganic phosphate (●). The release of a-p4 was measured by mixing the following solutions at the indicated temperatures: [500 nM DnaK, 4 μM ADP, 200 nM a-p4, 25 mM phosphate] + [200 nM GrpE, 5mM ATP]. The numerical values are given in Table III.

The latter measurement corresponds to the conditions during the steady state of the chaperone cycle, in which inorganic phosphate is generated *in situ* by the hydrolysis of ATP. The rate of the GrpE-facilitated R→T conversion, both in the presence and absence of phosphate, deviated from Arrhenius temperature dependence by not being faster or even somewhat slower at 45°C than at 37°C (Figure 6A and B). The rate of MABA-ADP/ATP exchange at 25°C was linearly dependent on the concentration of GrpE from 0.1 μM to 1 μM (not shown).

Increased sequestering of protein substrate under heat-shock conditions – The fraction of substrate sequestered in high-affinity R-state DnaK is conjointly controlled by DnaJ and GrpE (Figure 1). While the rate of the DnaJ-stimulated T→R conversion increases exponentially

with increasing temperature (Figure 5B), the rate of the GrpE-facilitated R→T conversion increases less and less with increasing temperature and even decreases at temperatures above 40°C (Grimshaw et al., 2001; see also Figure 6B). The rates of the DnaJ-stimulated T→R conversion (Figure 5A and B) and the GrpE-facilitated R→T conversion (Figure 6A and B) served to calculate the fraction of total DnaK existing as high-affinity (ADP·DnaK)·substrate complex in the case of RepA, RCMLA or pp at 25°C, 37°C and 45°C (Table V). Two effects were observed: first, the *cis*-effect of DnaJ resulted at all temperatures in a 5 and 9 times higher fraction of (ADP·DnaK)·RepA complex in the presence of 2.5 μM and 5 μM RepA, respectively, as compared to the fraction of high-affinity R-state DnaK in the absence of substrate, while RCMLA and pp as substrates only slightly increased the fraction of R state at all temperatures; secondly, the thermosensor control of GrpE resulted in a fraction of R-state DnaK about twice as high at 45°C than at 25°C, the thermosensor effect being independent of the presence and absence of any substrate. Combined, the *cis*-effect of DnaJ (at 5 μM RepA) and the thermosensor control of GrpE (at 45°C) increased the fraction of high-affinity R-state DnaK by one order of magnitude over that with pp or RCMLA as substrates at 25°C (Table V).

Table V Fraction of DnaK in the high-affinity R state. The fraction F of total DnaK in the high-affinity (ADP·DnaK)·substrate complex, i.e. [R state]/([R state] + [T state]) was calculated from the rate k'_{TR} of the DnaJ-stimulated T→R conversion and the rate k'_{RT} of the GrpE-facilitated R→T conversion: $F = k'_{TR}/(k'_{TR} + k'_{RT})$. The k'_{TR} values, determined by single-turnover ATPase activity measurements, and the k'_{RT} values, determined by measuring the release of a-p4 from ADP-liganded DnaK in the presence of inorganic phosphate, were taken from Figure 5A and 6B, respectively.

	25°C	37°C	45°C
Substrate	Fraction of R-state DnaK		
	% of total		
RepA (5 μM)	44	53	76
RepA (2.5 μM)	31	41	59
RCMLA (5 μM)	7.5	7.8	14
pp (5 μM)	6.7	9.0	19
None	5.0	7.6	13

Discussion

Binding and release of protein substrates – In this study, we explored the kinetics of the DnaJ/GrpE-controlled DnaK chaperone cycle with proteins, the physiological substrates, both under normal and heat-shock conditions. To follow the complex formation of DnaK with protein substrates in real-time, we labeled DnaK H541C with the thiol-reactive fluorophore acrylodan. The values of the kinetic and thermodynamic parameters of the formation of (ATP·a-DnaK H541C)·pp complexes correspond with those measured with unlabeled DnaK and a-pp. The two procedures for monitoring the interaction of DnaK with peptide substrates thus mutually validate one another. At all temperatures, the values of the binding parameters of ATP·a-DnaK H541C for RepA and RCMLA were found to be of the same order of magnitude as the values for pp and other DnaK-binding peptides reported previously (Pierpaoli et al., 1998). The values of k_1 and k_{-1} obtained with short peptides are sequence-specific and lie in a relatively broad range; hence, the differences between the protein substrates RepA or RCMLA and the peptide substrate pp cannot be interpreted as to reflect a fundamental difference in complex formation of proteins and peptides with DnaK. Apparently, there is no significant steric hindrance for complex formation with these protein substrates and no notable interaction site other than the peptide-binding cleft. The DnaK-binding properties of a protein substrate are thus primarily defined by the amino acid sequence of the segment that interacts with the peptide-binding site of DnaK. This notion is consonant with the crystal structure (Zhu et al., 1996) and solution structure (Landry et al., 1992) of the substrate-binding domain of DnaK, which was found to bind hydrophobic 7-residue segments of the substrate in extended conformation.

Non-native-protein sensor DnaJ – Due to the *cis*-effect of DnaJ, the fraction of RepA being fed into the chaperone cycle is substantially higher than that of RCMLA or pp, which both are

substrates that do not form ternary complexes and therefore cannot elicit the *cis*-effect of DnaJ. The kinetic partitioning between dissociation of the (ATP·DnaK)·substrate complex and its conversion to a high-affinity (ADP·DnaK)·substrate complex provides a sorting mechanism for substrates. DnaJ acts as sensor for non-native proteins; only proteins with at least two exposed hydrophobic stretches elicit its *cis*-effect and trigger the ATP-consuming chaperone action (Figure 7). The sorting of (ATP·DnaK)·substrate complexes includes, of course, not only the *de novo* formed complexes but also T-state complexes generated from R-state complexes by ADP/ATP exchange, the alternative for these complexes being another round in the chaperone cycle or release into the solvent. The triage by DnaJ prevents the loading of the chaperone system with proteins or protein fragments that do not or no longer need chaperone assistance and minimizes futile ATP consumption.

DnaJ and other Hsp40 that share the J domain essential for the interaction with Hsp70 proteins are found both as soluble and as membrane-anchored proteins. In contrast to the soluble Hsp40 homologs, the membrane-anchored homologs such as DjlA in *E. coli*, Sec63p in yeast endoplasmic reticulum and Mdj2p in yeast mitochondria do not have a substrate-binding domain. In analogy to the substrate-binding domain of soluble Hsp40, which serves to form ternary complexes with non-native protein substrates, anchoring of Hsp40 homologs to the membrane restricts the ATP-consuming chaperone action to the segment of the polypeptide chain emerging from the translocation channel. Similarly, uncoating of clathrin-coated vesicles might be triggered by bringing Hsc70 and auxilin (Hsp40) into close proximity upon attaining the distinct geometry of the completely assembled coat.

Thermosensor GrpE – The rate of GrpE-facilitated ADP/ATP exchange proved to be substrate-independent and only controlled by temperature. With increasing temperature, melting of the long helix pair in the GrpE dimer decreases the efficacy of GrpE in catalyzing nucleotide exchange (Grimshaw et al., 2001, 2003; Gelinas et al., 2002, 2003). At heat shock,

the reduced activity of GrpE has recently been shown to increase the fraction of peptide substrate sequestered by DnaK (Siegenthaler et al., 2004) and indeed has proved advantageous for protecting protein substrates *in vitro* against both heat denaturation and aggregation (Siegenthaler and Christen, 2005).

The DnaK system in *E. coli* is not the only chaperone system that takes advantage of direct thermal control. GrpE in the thermophilic eubacterium *Thermus thermophilus* and Mge1p, a GrpE homolog in yeast mitochondria, have also been found to undergo a reversible thermal transition within the physiological temperature range (Groemping and Reinstein, 2001; Moro and Muga, 2006). Different from the melting of the helix pair in the *E. coli* GrpE dimer, the thermal transition in *T. thermophilus* GrpE affects the β–sheet domain and in Mge1p results in dimer dissociation. Direct adaptation to heat shock was also found in small heat shock proteins, which at heat shock temperatures undergo reversible conformational changes that improve their chaperone efficiency; responses of this kind have been reported for yeast Hsp26 (Haslbeck et al., 1999), wheat Hsp16.9 (van Montfort et al., 2001) and rat Hsp22 (Chowdary et al., 2004) as well as for the endoplasmic reticulum-resident chaperone calreticulin (Rizvi et al., 2004). Hsp33 in *E. coli* is a redox-regulated chaperone, its activity being enhanced by reversible conformational changes upon oxidative stress (Jakob et al., 1999).

Direct heat shock response through conjoint action of DnaJ and GrpE – If cells are subjected to heat shock conditions, the concentration of non-native proteins exposing hydrophobic binding sites for DnaK and DnaJ will increase. The rate of the DnaJ-stimulated ATP hydrolysis increases exponentially with increasing temperature and in the presence of unfolded proteins is additionally accelerated by the *cis*-effect of DnaJ.

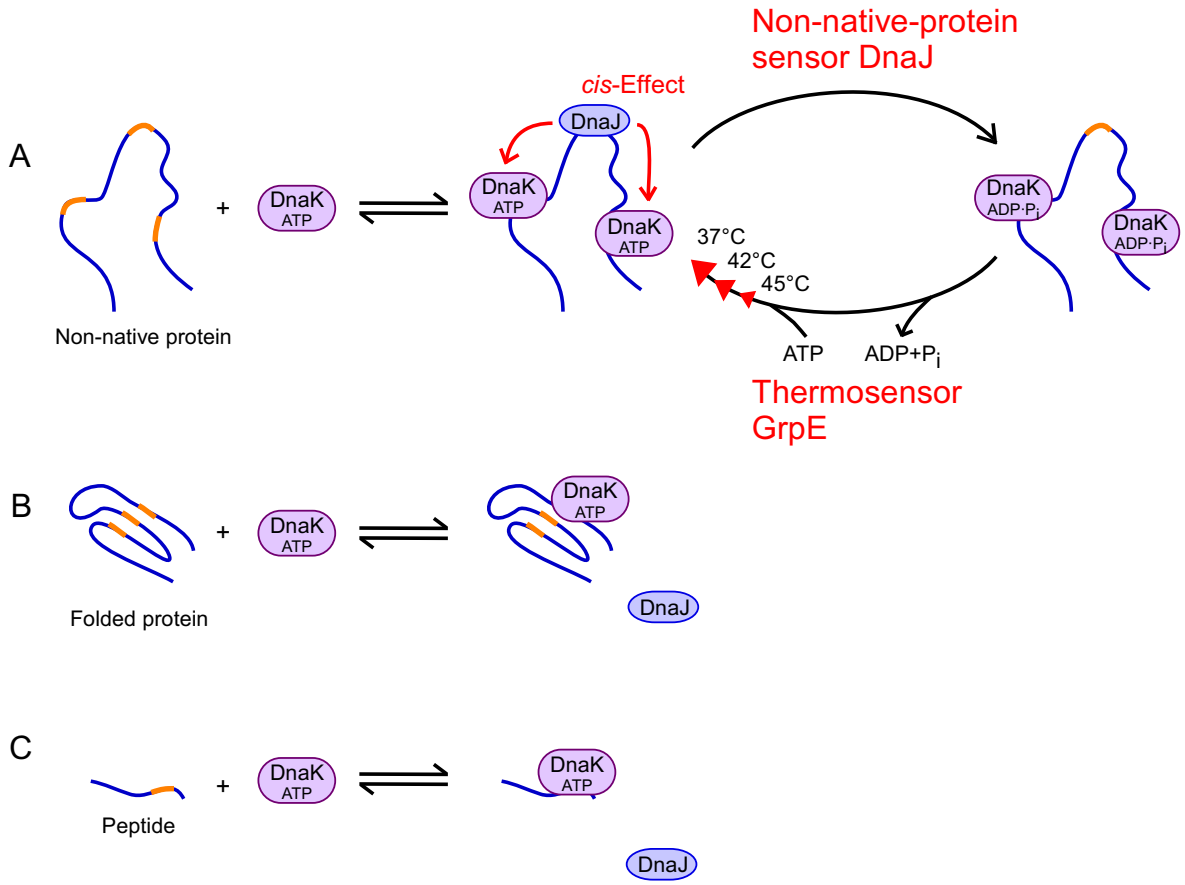

Figure 7 DnaK/DnaJ/GrpE chaperone cycle and its direct modulation by heat shock. (**A**) DnaJ with its *cis*-effect acts as a substrate-sorting device and preferentially feeds non-native proteins with at least two exposed binding sites for DnaK or DnaJ into the cycle. Binding sites for DnaK or DnaJ on polypeptide chains occur on average every 36 residues (Rüdiger et al., 1997). (**B**) Native proteins and (**C**) peptides that expose only one DnaK/DnaJ-binding site have a greatly reduced chance of entering the cycle because the rapid dissociation of the (ATP·DnaK)·substrate complexes prevails over the slow conversion into high-affinity (ADP·DnaK)·substrate complexes. Upon heat shock, the rate of the DnaJ-stimulated formation of high-affinity (ADP·DnaK)·protein complexes increases exponentially with increasing temperature and is additionally accelerated by the *cis*-effect of DnaJ in the case of non-native protein substrates. The rate of the GrpE-facilitated conversion of the high-affinity (ADP·DnaK)·protein complexes into low-affinity complexes increases less and less with increasing temperature and even decreases above 40°C. During heat shock, the conjoint action of non-native-protein sensor DnaJ and thermosensor GrpE promotes dynamic sequestering of aggregation-prone proteins in high-affinity (ADP·DnaK)·protein complexes.

The higher the concentration of non-native proteins and thus the higher the concentration of ternary (ATP·DnaK)·substrate·DnaJ complexes the more pronounced the *cis*-effect will be (compare the rates of the T→R conversion at 2.5 µM and 5 µM RepA; Figure 5A). Hence, the fraction of (ATP·DnaK)·protein complexes converted into high-affinity (ADP·DnaK)·protein complexes increases (Figure 7). The temperature-induced decrease in rate of GrpE-facilitated

ADP/ATP exchange in its turn retards the release of the protein substrate from DnaK. DnaJ as sensor for non-native-proteins and GrpE as thermosensor thus conjointly promote dynamic sequestering of the protein substrate as high-affinity complexes with ADP·DnaK, the ensuing decrease in concentration of free non-native proteins preventing their aggregation.

The DnaK/DnaJ/GrpE chaperone machinery exerts two seemingly distinct functions, i.e. promoting protein folding and preventing protein aggregation. Both processes rely on a continuously running ATPase cycle. Due to the thermosensor control of GrpE, the overall rate of the DnaK ATPase cycle at 45°C is similar to that at 37°C (not shown). The steady-state R/T ratio of DnaK, however, is shifted toward the R state at heat shock temperatures. Apparently, sequestering of substrate by DnaK becomes more important at higher temperatures, which are unfavorable for folding. This temperature-dependent shift in the operational mode is consistent with the notion that the chaperone effect of the DnaK system might result from two different mechanisms (Pierpaoli et al., 1997; Mayer et al., 2000; Slepenkov and Witt, 2002; Ben-Zvi et al., 2004): “active” folding assistance by performing conformational work on the substrate and “passive” sequestration of hydrophobic stretches of the polypeptide chain. Depending on temperature, the two modes might contribute to a varying degree to the chaperone effect. Non-native-protein sensor DnaJ and thermosensor GrpE adapt the operational mode of the DnaK system to the particular situation and optimize the chaperone effect. GrpE directly senses heat shock by a temperature-induced conformational change, while DnaJ indirectly senses heat shock or other cellular stress by the ensuing misfolded proteins.

Similar to the regulation of key enzymes in metabolic pathways, the chaperone action of the DnaK/DnaJ/GrpE system is thus not only controlled by changing the concentration of the chaperones but also by direct modulation of its activity. Moreover, and again in analogy to metabolic regulation, the instant and reversible response to elevated temperature bridges the time lag of the σ^{32}-mediated heat shock response and its reversal.

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